An attempt at an epidemiological explanation

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THE INCREASED INCIDENCE OF MENINGOCOCCAL DISEASE IN THE NETHERLANDS 1980-1990

An attempt at an epidemiological explanation

ACADEMISCH PROEFSCHRIFT

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CHAPTER 1

GENERAL INTRODUCTION

Meningococcal disease (MD) is a serious illness caused by *Neisseria meningitidis* (meningococcus). The clinical spectrum of MD ranges from a relatively harmless flu-like syndrome to meningitis and fulminant septic shock with fatal outcome. The occurrence of cases of MD in rapid succession generally leads to public alarm. Because of the severity of the disease and the threat of an epidemic, MD must be notified to the health authorities in many countries.

Since 1958, isolates of *N. meningitidis* recovered from patients with MD in the Netherlands have been forwarded to the Netherlands Reference Laboratory for Bacterial Meningitis (RLBM) of the Department of Medical Microbiology, University of Amsterdam, and the National Institute for Public Health and Environmental Protection by almost all Dutch clinical microbiological laboratories. The isolates are stored at -70°C, and are readily available for further research. The collection currently comprises over 7000 meningococcal isolates, and is of great value for fundamental and epidemiological research. From 1982 onwards, a gradual increase in the number of submissions of meningococcal isolates to the RLBM was noted, and in 1988 the incidence of MD in the Netherlands reached a sub-epidemic level. In 1989 it was decided to conduct a nation-wide survey on MD. This thesis addresses various epidemiological aspects of this survey. In this introductory chapter some general features of *N. meningitidis* and MD are described, as well as aspects of the prevention of MD. Finally, the objectives of the study and the contents of this thesis are indicated.

Neisseria meningitidis

Bacteriology

N. meningitidis is a gram-negative diplococcus, which can be directly visualized in gram-stains of the cerebrospinal fluid, fluid from petechial lesions or the buffy-coat of the peripheral blood of infected patients.[1] The organism grows easily on various media, such as blood agar and chocolate agar.[1] Optimal growth is achieved in a moist environment at 35-37°C under an atmosphere of 5-10% CO_2.[1] *N. meningitidis* can be distinguished from other *Neisseria* species, like *Neisseria lactamica* and *Neisseria gonorrhoeae*, on the basis of its sugar metabolism.[1]

Among the principal surface structures of *N. meningitidis* are pili, or fimbriae, the capsule and the outer membrane.[1,2] Pili are finger-like surface appendages which play an important role in the attachment of *N. meningitidis* to the mucosal surface of the nasopharynx. Pili show marked intra and interstrain variability, both quantitatively and qualitatively. The majority of meningococci isolated from patients with MD produce a polysaccharide capsule, which is considered as a virulence property, because it allows the meningococcus to evade host defense mechanisms and to survive in the bloodstream of the host.[3] Directly underneath the capsule lies the outer membrane, which contains a number of proteins and lipopolysaccharides.[2,4,5] Based on their molecular weights, the major outer membrane proteins (OMPs) are categorized

into 5 distinct classes.[6] The class 1 OMP is quantitatively variable, and some meningococci do not express a class 1 OMP.[7] Every meningococcus has either a class 2 OMP or a class 3 OMP (mutually exclusive), which are quantitatively the major OMPs.[7] Both the class 1 OMPs and the class 2/3 OMPs are surface-exposed, as is the class 5 OMP.[7] A single meningococcus can express one or more class 5 OMPs, which are qualitatively highly variable, or no class 5 OMP at all.[7] The class 1, 2 and 3 OMPs show less qualitative differences, and because they are surface-exposed they can be used for the characterization of meningococci (see next paragraph).[8] The class 4 OMP, which is always present, is not surface-exposed and appears to be highly conserved.[7] The lipopolysaccharides (LPS), also known as endotoxins, form a major constituent of the meningococcal outer membrane.[7,9] They play an important role in the development of endotoxic shock.[10]

Surface characterization

Antigenic differences in the capsules, class 1 OMPs, class 2/3 OMPs and LPS are used for the characterization (phenotyping) of meningococci.[8]

Meningococci are classified into serogroups on the basis of antigenic differences of the capsular polysaccharide. The serogroup can be determined by microprecipitation.[11] Currently, 12 different serogroups are distinguished, indicated by a capital letter (Table 1).[1,12] The serogroups H, I, K and L have never been isolated from patients,[1] and are not determined in the RLBM. Meningococci that do not have a capsule are consequently nongroupable.

Table 1. Surface structures of *Neisseria meningitidis* used for meningococcal characterization (phenotyping)

Surface structure*	Designation	Labels
CPS	Serogroup	A, B, C, X, Y, Z, 29E, W-135 (H, I, K, L)†
Class 2/3 OMP	Serotype	1, 2a, 2b, 2c†, 4, 14, 15, 16
Class 1 OMP: VR1	Subtype	P1.5, P1.7, P1.12
Class 1 OMP: VR2	Subtype	P1.1, P1.2, P1.3†, P1.4, P1.6, P1.9, P1.10, P1.14, P1.15, P1.16
LOS	Immunotype	L1 through L11

* CPS = capsular polysaccharide; OMP = outer membrane protein; VR = variable region; LOS = lipooligosaccharide

† not determined in our laboratory

Antigenic variation in the class 2/3 OMPs and class 1 OMPs forms the basis for serotyping and subtyping, respectively.[8] Serotypes and subtypes are designated by an arabic number, but the number of the subtype is preceded by the prefix "P1" (Table 1). Both serotyping and subtyping can be performed in a whole-cell ELISA using monoclonal antibodies (moabs).[13] Some meningococci, however, do not react with the currently available set of moabs, and they are labelled non(sub)typeable. These strains are of a yet unidentified serotype or subtype. Meningococci that do not express a class 1 OMP are consequently nonsubtypeable. The number of new specific moabs is still growing, resulting in the recognition of an increasing number of serotypes and subtypes. The panel of moabs used in the RLBM until 1989 included 7 moabs for serotyping and 7 moabs for subtyping. In 1989, moabs against the subtypes P1.4, P1.10, P1.12 and P1.14 were developed. These moabs, which considerably reduced the proportion of nonsubtypeable strains, are also included for the meningococcal subtyping described in this study. The moab against subtype P1.5 became available in 1992, and is used in only part of the study. At the end of 1992, a moab against subtype P1.13 was found, but this moab has not been incorporated in the panel of moabs used for subtyping in this study.

It has recently been demonstrated that the class 1 OMP harbours 2 variable regions (VR1 and VR2) each determining a distinct set of subtypes.[14 15] The subtypes that have been recognized to date are determined by epitopes, either of VR1 (P1.5, P1.7 and P1.12) or VR2 (the remaining subtypes). Therefore, meningococci that have a class 1 OMP are characterized by a combination of subtypes of the 2 different variable regions. Well-known subtype combinations are P1.7,1 and P1.7,16.[16-20] Subtype P1.5 of VR1 is often found in combination with P1.2 of VR2 (P1.5,2).

Molecular variation in the oligosaccharide portion of the LPS of the meningococcal outer membrane forms the basis for immunotyping.[4 5 8] LPS immunotyping, therefore, is more properly labelled as lipooligosaccharide (LOS) immunotyping.[21] Currently, 11 different immunotypes are distinguished: L1 through L11 (Table 1).[4 5] Recently, 2 new immunotypes, L12 and L13, were identified, but their true existence has not yet been established.[22] Many meningococcal strains express more than one immunotype.[4 5] In the past the determination of the immunotype has been difficult, due to the complexity of the detection methods involved.[4 5] In the last few years an increasing number of LOS-specific moabs have been developed,[23-25] which enables the determination of immunotypes in a whole-cell ELISA, thus facilitating immunotyping (Chapter 4).

A meningococcal isolate is characterized by the combination of serogroup, serotype, subtype and immunotype.[8] This combination is called a phenotype. Examples of phenotypes are B:2b:P1.2:L2, B:4:P1.4:L3, and B:15:P1.7,16:L3,8. As indicated above, meningococci that have a class 1 OMP are characterized by a subtype combination. However, most meningococci are found to have a single subtype of either one of the 2 variable regions of the class 1 OMP and, therefore, must be considered as partially nonsubtypeable.[17-19 26] Though being part of the full character-

ization of *N. meningitidis*, immunotyping is not regularly performed and the immunotype is often omitted from the phenotype description.

Phenotyping has proved to be a valuable tool for studying the spread of MD.[17 19 22 25-37] For the future prevention of MD by vaccination, monitoring of the changes in the distribution of serotypes and subtypes will become of utmost importance (see below).

Genotyping

In order to assess the genetic relationship of different meningococcal isolates, various genotyping methods are available. Common methods are multilocus enzyme electrophoresis and restriction fragment length polymorphism.[38 39]

In multilocus enzyme electrophoresis, also called electrophoretic typing, the pattern of isoenzyme variants of several common cytoplasmic enzymes is used to estimate the genetic content of meningococcal isolates. Isoenzyme variants are determined by using specific enzyme stains after starch gel electrophoresis.[38] Based on the results of the electrophoretic migration of each enzyme, each isolate is then assigned a multidigit score. Each multidigit score thus obtained forms an electrophoretic type (ET), designated by an arabic number.[38] The various ETs are labelled from 1 to the total number of different ETs detected among the isolates tested. The number of different ETs, therefore, depends on the number of isolates. Thus, the designation of ETs is nominal and varies from survey to survey, and ET numbers allocated in different surveys usually are not similar or exchangeable, unless the ET numbers of one survey are adapted to those of another. Each separate ET represents a clone with a particular genetic background. On the basis of the isoenzyme variants of the various enzymes, the genetic relationship of the ETs of the tested isolates is assessed by the use of multivariate statistical methods,[38] and clusters (or lineages) of clones that are genetically closely related can be distinguished.[22 25 34 35 37 40-43] Electrophoretic typing has been applied for the determination of the genotype of meningococcal isolates in the Netherlands.[43]

Restriction fragment length polymorphism (RFLP) is another method for the assessment of genetic relationship of meningococci.[39] The procedure involves extracting cellular DNA and fragmentating it with restriction endonucleases. The fragments are subsequently separated by electrophoresis. Following denaturation of the DNA, the fragment bands are blotted. After hybridization with a DNA probe, the fragments which contain the sequence to which the probe is directed are visualized. By the use of the probe, a reduction in the number of DNA bands is obtained, which facilitates the interpretation of the DNA patterns. The choice of the probe is based on repeated DNA sequences found in various parts of the chromosome. In order to be able to characterize all possible meningococcal strains, it is essential to select a probe with broad specificity. RFLP types are designated according to the various RFLP patterns found in the study under consideration, and the number of different RFLP types also depends on the number of isolates tested. RFLP results are similar to those obtained

by electrophoretic typing.[39] RFLP typing has not been used for genotyping of meningococcal isolates in the Netherlands.

Phenotypic similarity does not always imply genotypic similarity, and isolates that are genetically closely related may be of different phenotypes.[39-43] A newly-appearing meningococcal strain in a certain geographical area, however, is often homogenous with regard to both the genotype and phenotype during the first few years after its appearance.[41 43]

Meningococcal disease

Epidemiology

The natural habitat of the meningococcus is the human nasopharynx.[2] The bacterium is transmitted from person to person by small droplets, and colonization usually leads to an asymptomatic carrier state, and occasionally to a clinical syndrome of meningitis or septicemia.[1 2 44 45] Asymptomatic carriers are the main source of infection.[1 45] Carrier rates in the open population range from 5% to 11%.[44 46-49] The serogroups B, C, Y and W-135 are common among carriers, and meningococci of the serogroups X, Z and 29E, as well as nongroupable (nonencapsulated) meningococci, are found almost exclusively among carriers.[44 46 49] Carriage of meningococci of serogroup B is usually characterized by long duration (median 9 months) and low acquisition rates (i.e. the incidence of carriage).[1 48] Serogroup A, however, has been found to be less prevalent among carriers, but has demonstrated high acquisition rates and a rapid turn-over.[1 47 48]

Meningococcal disease is endemic in both developing and industrialized countries.[44] In western Europe most cases of MD are reported during the winter months, but in (sub)tropical areas the incidence of MD is highest during or at the end of the hot, dry season.[1 44 50] Desiccation of the mucosal surface during these periods has been assumed to facilitate the invasion of nasopharyngeal meningococci.[1] In most developing countries the majority of the cases of MD is due to meningococci of serogroup A, whereas in Europe and the Americas the serogroups B and C prevail.[17 25 26 28-37 44 50-54] The uncommon serogroups (X, Y, Z, 29E, W-135) and nongroupable isolates are considered as opportunistic pathogens, because they seem to cause disease predominantly among predisposed patients, e.g. immuno-compromised patients.[53 55] During endemic periods many different phenotypes and clones are found among serogroup B and C isolates.[17 26 30 31 35 43] These two serogroups share many serotypes, subtypes and immunotypes.[4 5] Serogroup A isolates are rather homogenous with regard to both the phenotype and genotype.[22 25 34 37 56]

Epidemics of MD have been reported regularly world-wide.[25 28 30 34 36 37 44 50 51 54 57-61] Epidemics are mainly due to a particular meningococcal clone or a limited number of closely related clones.[25 34 36 37 41 43 54 60 61] In most developing countries epidemics are usually caused by meningococci of serogroup A, but in Europe and the Americas by meningococci of the serogroups B or C. The appearance of a new meningococcal

clone with an new phenotype (B:2b:P1.2) was responsible for the 1966 epidemic in the Netherlands.[43 62] Meningococci of the phenotype B:15:P1.7,16 caused a protracted epidemic in Norway that started in 1974.[51 63] This phenotype was the main representative of a complex of meningococcal clones (ET-5 complex), which has slowly spread to the south of Europe, and further to the Americas, but epidemics or local outbreaks in other parts of the world, due to clones of the ET-5 complex, were often of other phenotypes, e.g. B:4:P1.15 in Cuba and B:15:P1.3 in Chile.[41]

Natural immunity

Infants younger than 6 months of age are relatively protected against MD, due to passive immunization by transplacentally transferred maternal antibodies.[64] As the level of these antibodies decreases, the host will become susceptible to various meningococci, and natural immunity has to be built up.[64] Immunity is thought to be acquired by (repeated) carriage of both encapsulated and nonencapsulated meningococci, as well as nonvirulent *Neisseria* species, like *N. lactamica*.[64] Both specific and cross-reactive antibodies are induced by the various surface components of these meningococci. During a life-time many different meningococci will be acquired, resulting in broad immunity against various meningococcal antigens.

Age-distribution

MD is predominantly a disease of childhood, and the chance of its occurrence is inversely related to the level of specific antibodies in the host.[64] The peak incidence is among children from 6 months to 5 years of age who are immunologically naive, and another small peak is noted among teenagers.[44 65 66] The latter peak might be due to a change in life-style or biological changes during puberty.[67]

The age-distribution differs among the various serogroups.[44 53 65] Meningococci of serogroup C, and to a lesser extent of serogroup A, are found relatively more often among older patients, as compared to those of serogroup B.

The emergence of a new meningococcal phenotype in a certain geographical area often results in an increase in the number of cases in the older age-categories, which are then relatively over-represented.[65 68 69]

Pathogenesis and clinical syndromes

A review of current knowledge of the pathogenesis of bacterial meningitis has been provided recently by Quagliarello and Scheld.[3] The pili are thought to play a main role in the adherence on cells present in the nasopharyngeal mucosa and the blood-brain barrier. The capsule enables the meningococcus to evade the host defense mechanisms and to survive in the bloodstream of the host. The LOS (endotoxin) is considered as an important mediator in the inflammatory process in the CSF and in the development of septic shock.[3 10] Meningococci are capable of releasing parts of their outer membrane ("blebs"), which contain high concentrations of LOS.[2 3] LOS induces a cytokine cascade (tumor necrosis factor, interleukin-1, interleukin-6), which

is assumed to be the main trigger of septic shock and diffuse intravascular coagulation.[3 70]

After entering the bloodstream of the host, the meningococcus may cause a variety of clinical syndromes ranging from a benign flu-like disease ("chronic meningococcemia") to fulminant septicemia with septic shock and multiple organ failure.[1 2 44 53 71 72] The most common clinical manifestation of MD, however, is meningitis, but in 8-18% of the cases septicemia is the sole manifestation.[28 52 53 58 73-75] In septicemia severe hemodynamic disturbances and coagulopathy (diffuse intravascular coagulation) may occur, characterized by pronounced shock, renal insufficiency, cardiac failure, adult respiratory distress syndrome, and massive bleeding in many organs, such as the adrenal glands, gastrointestinal tract and cerebrum.[1 72]

Case-fatality rate and sequelae

The crude case-fatality rate (CFR) of MD ranges from 2-14%.[17 28 36 44 50-53 57-59 66 73-76] Fatalities may be directly due to cerebral damage (cerebral infarcts and herniation due to brain edema), but result more often from pronounced endotoxic shock.[1 3 70] Depending on the definitions and circumstances the reported CFRs among meningitic patients range from 1-11%, whereas those among septicemic patients range from 9-41%.[36 44 50 52 53 58 66 74 75] Despite the improvement of clinical care during the past 4 decades, the CFR of MD has remained essentially unchanged.[76]

Of the patients who do recover, 3-13% are left with severe sequelae.[28 44 52 53] Sequelae may result directly from infective processes in the meninges or from hemodynamic and hemostatic complications.[1] Common sequelae are loss of hearing or even complete deafness, severe skin necrosis leading to scars and contractures or necessitating amputation, paralysis of a cranial nerve, hemiparesis, seizures, and mental retardation.[28 44 53]

Several surface characteristics of *Neisseria meningitidis*, such as the serogroup and serotype, have been found to be associated with an unfavourable outcome of MD.[52 53 77] However, the results of these reports were conflicting. For example, in Scotland from 1972-1982 more fatalities of MD were found after disease due to isolates of the serogroups A and C, as opposed to those of the serogroups B and W-135,[52] whereas in a survey in the Netherlands from 1959-1981 this association was reversed, isolates of the serogroup W-135 and the uncommon serogroups showing the highest CFRs, followed by those of the serogroups B, C and A.[53] This suggests that the observed associations may have been confounded by other factors, either of the host or the pathogen.

Prevention of meningococcal disease

Chemoprophylaxis

Intimate contacts of patients with MD, such as the household contacts, have an increased risk of contracting MD.[78-80] To protect these contacts, chemoprophylaxis is

prescribed in many countries, among which the United States and Great Britain.[81 82] In the Netherlands, however, the prescription of chemoprophylaxis is not accepted practice. Rifampicin is the drug of first choice, and alternatives are minocycline, ceftriaxone and ciprofloxacin.[81 82] These drugs produce sufficient salivary concentrations to ensure the elimination of carrier strains.[83-85] It is assumed that by eliminating meningococci from the nasopharynx of the household contacts the transmission of meningococci within the household will be interrupted, and secondary cases of MD will be prevented. However, the beneficial effect of these drugs for the prevention of secondary disease has not been evaluated in randomized trials.

Immunoprophylaxis

For protection against MD due to meningococci of the serogroups A, C, W-135 and Y, capsular polysaccharide vaccines are available.[86] These vaccines elicit serogroup-specific immunity in older children and adults, but the immune-response in children under 2 years of age is poor, especially response to the C polysaccharide. In addition, because of their T-cell independency, these vaccines do not induce immunological memory, and regular revaccination is necessary. Experimental research has shown that the performance of capsular vaccines is improved by conjugating the polysaccharide of the vaccine to protein, e.g. tetanus toxoid.[87] These conjugate vaccines are also immunogenic in infants and induce immunological memory. The efficacy of the vaccines, however, has not yet been evaluated in humans.

To date, no effective vaccine is available for protection against disease due to meningococci of serogroup B, the most prevalent serogroup in developed countries. The B polysaccharide has proved to be poorly immunogenic in humans, which might be due to its close resemblance to fetal human brain tissue,[88] and other serogroup B vaccine potentials are now being investigated. The principal candidates are the class 1 OMPs and class 2/3 OMPs of the meningococcus.[89] Experimental vaccines that consist of outer membrane vesicles (OMVs) containing these OMPs have been developed.[86 89] The antibodies elicited by the OMPs of these vaccines are subtype and serotype-specific.[89] The composition of the experimental vaccines recently tested in Norway, Chile and Cuba[90-92] was based on the serotype and subtype of the most prevalent serogroup B phenotype in these countries: B:15:P1.7,16 in Norway, B:15:P1.3 in Chile and B:4:P1.15 in Cuba. In many countries, however, the serogroup B meningococci show marked heterogeneity with regard to serotype and subtype,[17 26 31 75] and for those countries the above-mentioned vaccines are only of limited value. In order to ensure broad specificity, further research is directed towards the development of multivalent serogroup B OMV vaccines, guided by the local distribution of serotypes and subtypes.

Components of the oligosaccharide structure of the meningococcal LOS are other potentials for a vaccine against serogroup B MD.[86 89] Experimental conjugate vaccines of synthetic oligosaccharide structures coupled to meningococcal OMP are now being tested in animals.[93]

Study objectives and contents of this thesis

From 1982 onwards there was a gradual increase in the number of submissions of meningococcal isolates to the RLBM. This increase seemed to stabilize in 1986, but at the end of 1988 the number of submissions increased sharply, and the onset of an epidemic of MD in the Netherlands was feared.

At the beginning of 1989 it was decided to conduct a nation-wide survey on MD. The main objective of the study was to find explanations for the increased incidence of MD in the Netherlands in the 1980s and to provide data which could assist the further development of a serogroup B vaccine. Further goals were the assessment of the secondary attack rate of MD among the household contacts of primary patients and the determination of the prescription of chemoprophylaxis to these contacts for the prevention of secondary disease.

The survey started on 1st April, 1989, and data collection was co-ordinated by the Institute for Research in Extramural Medicine (EMGO Institute) of the Vrije Universiteit in Amsterdam. Epidemiological data and serum samples were collected from patients with MD, their family members and healthy controls. This was made possible thanks to the intense efforts of many public health officers, medical microbiologists and attending physicians. For the further analysis of secular changes in the characteristics of *N. meningitidis* in the past 32 years, the extensive collection of meningococcal isolates of the RLBM was available.

Serological research still continues, and the serological aspects of this survey will be reported elsewhere. This thesis presents other epidemiological aspects of the study. Chapter 2 describes the changes in the distribution of the various surface markers of *N. meningitidis* in the Netherlands from 1958 to 1990. Chapter 3 deals with the phenotypic and genotypic changes that occurred in a new meningococcal clone complex that appeared in 1980 in the Netherlands. In Chapter 4 an algorithm is presented for the assignment of the LOS immunotype, as determined in a whole-cell ELISA using monoclonal antibodies. Chapter 5 contains a multivariate analysis of the association between surface characteristics of *N. meningitidis* and host characteristics, on the one hand, and the outcome of MD on the other. Chapter 6 describes the occurrence of secondary cases of MD among the household contacts of primary patients during the period 1989-1990, and the prescription of chemoprophylaxis in the Netherlands. In the final chapter, the results are discussed in conjunction with each other, and recommendations are made for further research.

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CHAPTER 2

MENINGOCOCCAL DISEASE IN THE NETHERLANDS, 1958-1990

A steady increase in the incidence since 1982 partially caused by new serotypes and subtypes of *Neisseria meningitidis*

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ABSTRACT

In order to explain a three-fold increase in the incidence of meningococcal disease in the Netherlands during the 1980s, we serotyped and subtyped *Neisseria meningitidis* isolates recovered between 1958 and 1990 from >3000 patients with systemic disease. No single strain could be held responsible for the increase. Apart from the newly-introduced strain B:4:P1.4, which became the most prevalent phenotype in 1990 (21% of all isolates), the majority of the cases in 1990 were caused by many different strains that were already present in the Netherlands before 1980. For the period 1980-1990, a shift in the age-distribution of patients with meningococcal disease from younger to older age-categories was found, particularly with regard to cases due to meningococci of serogroup B; this shift is explained by the changing distribution of serotypes and subtypes within serogroup B. A multivalent serogroup B, class 1 outer-membrane-protein vaccine of a stable composition could theoretically have prevented 80% of all serogroup B meningococcal infections in the Netherlands during the past 30 years.

INTRODUCTION

Meningococcal disease (MD) still poses major health problems in both developing and industrialized countries. Before 1980 the annual incidence of MD in the Netherlands varied between 0.7 and 2.0 cases per 100,000 inhabitants, whereas epidemics that occurred in 1946 and 1966 were associated with an annual incidence of 9.9 per 100,000 and 4.1 per 100,000, respectively.[1 2] During the 1980s the incidence of MD in the Netherlands gradually increased and it reached 3.5 per 100,000 inhabitants in 1990.

Meningococci are classified into serogroups, serotypes and subtypes on the basis of antigenic differences in their capsular polysaccharides, class 2/3 outer-membrane proteins (OMPs) and class 1 OMPs, respectively. Studying the serotype and subtype distribution of meningococci contributes to the understanding of the epidemiology of MD.[3] The number of monoclonal antibodies used for serosubtyping is still growing, thus improving our insight into the spread of MD.[4] The characterization of the chromosomal genotype of *Neisseria meningitidis* isolates by multilocus enzyme electrophoresis is another valuable tool for studying the epidemiology of MD.[5]

Both in Europe and in the Americas, serogroup B is the predominant serogroup causing MD, followed in frequency by serogroup C.[6 7] Currently, no effective vaccine is available for protection against serogroup B MD. The development of such a vaccine is primarily focused on class 1 OMPs.[8 9] Experimental vaccines against serogroup B, which to some extent covered the local needs, were tested in Norway, Cuba and Chile.[10-12] The reported efficacy of these vaccines ranged from 51% to 81%. If OMP vaccines are to be used for the prevention of serogroup B MD, it becomes essential to monitor the distribution of subtypes over time in order to allow for adaptation of the vaccine to the current circulation of subtypes.

Since 1958, meningococcal isolates from blood and/or cerebrospinal fluid (CSF) have been submitted for further classification to the Reference Laboratory for Bacterial Meningitis (RLBM) in Amsterdam by almost all microbiologists in the Netherlands. The strain collection currently comprises of approximately 7000 meningococcal isolates. On the basis of this extensive collection, we report the changing pattern of the serotype and subtype distribution of meningococci in the Netherlands, as determined with the use of monoclonal antibodies against 7 serotypes and 11 subtypes. Our typing results are compared with those obtained with multilocus enzyme electrophoresis in a former survey of strains from patients in the Netherlands.[13] Special attention is paid to the period 1980-1990, during which the incidence of MD gradually increased to a subepidemic level. By comparing the results from typing in this period with those from the period 1958-1975, we attempt to explain the increased number of cases. In addition, we describe the shift in the age-distribution and the increase in the recovery of isolates from blood alone - phenomena that were observed during the last decade - and discuss their relation to the serogroup and serosubtype distribution.

MATERIALS AND METHODS

Bacterial isolates

Isolates of *N. meningitidis* that were recovered from the blood and/or CSF of patients with systemic MD in the Netherlands were sent on chocolate-agar slants to the RLBM by regional laboratories. Upon arrival, the isolates were stored at -70°C on glass beads as suspensions in 15% glycerol broth until further processing. Data on the source of isolation (CSF, blood or both) and on the patient (age and gender) were collected from the forms supplied by the laboratories. A case was defined as septicemic if *N. meningitidis* was cultured from blood alone. All isolates submitted to the RLBM from 1980 to 1990 were included in the general analysis.

Grouping and typing

Upon receipt of the strains in our laboratory, we performed serogrouping by means of Ouchterlony gel diffusion with use of antisera raised in rabbits against serogroups A, B, C, X, Y, Z, W-135 and 29E.[14]

For detection of changing patterns of serotypes and/or subtypes, all serogroup A, B and C meningococci obtained in 1980, 1983, and 1985-1990 (of which 11 isolates were lost) were serotyped and subtyped by means of a whole-cell ELISA with use of specific monoclonal antibodies against class 2/3 and class 1 OMPs of *N. meningitidis*.[4]

Table 1. Monoclonal antibodies and reference strains used for serotyping and subtyping of meningococcal isolates

Identification	Monoclonal antibody	Reference strain
Serotype		
1	MN3C6B	M1080
2a	MN2D3F	B16B6
2b	MN2C3B	2996
4	MN14G21.17	870227
14	MN5C8C	S3446
15	MN15A14H6	H44/76
16	MN93E 9-1	60E
Subtype		
P1.1	MN14C2.3	M1080
P1.2	MN16C13F4	B16B6 and 2996
P1.4	MN20B9.34	882066
P1.6	MN19D6.13	M990
P1.7	MN14C11.6	H44/76
P1.9	MN5A10F	M982
P1.10	MN20F4.17	870227
P1.12	MN20A7.10	S3032
P1.14	MN21G3.17	S3446
P1.15	MN3C5C	H355
P1.16	MN5C11G	H44/76

The panel of monoclonal antibodies, which were produced as previously described,[15] is shown in Table 1. Monoclonal antibodies specific for subtypes P1.4, P1.10, P1.12 and P1.14 have been developed recently and have not been described before. These antibodies were defined to be specific for class 1 OMPs by means of immunoblotting-analysis.

To analyse whether the serotypes and subtypes that appeared in the 1980s among serogroup B meningococci were newly-introduced in the Netherlands, we serotyped and subtyped all serogroup B isolates obtained in 1960, 1965, 1970, and 1975. Because many isolates from 1960 and 1965 had been lost in storage, typing was extended to serogroup B isolates obtained in adjacent years. The influence of long-term storage of isolates at -70°C on OMP typing was tested by comparison of the current typing results with those of isolates obtained between 1958 and 1981, which had already been screened for the presence of serotype 2a and 2b by filter radio-immunoassay and for serotype 15 by staphylococcal coagglutination.[16-18] Differences with former typing results were minor (6%), thereby indicating that long-term storage at -70°C probably has no major effect on OMP-typing.

To assess the association between genotypes and serosubtypes, as determined with the current extensive set of monoclonal antibodies, we serotyped and subtyped all serogroup B isolates obtained between 1958 and 1986, of which the electrophoretic type (ET) was determined by Caugant *et al.*[13]

Statistical methods

Statistics pertaining to the Dutch population were obtained from the Central Bureau of Statistics.

The χ^2 test was used for the analysis of contingency tables. In these analyses the serogroups X, Y, Z, W-135, and 29E and nongroupable isolates were combined because of their small numbers. For the same reason, the serotypes 1, 14, and 16 and the subtypes P1.1, P1.6, P1.7, P1.7,1, P1.9, P1.10, P1.12, and P1.14 among serogroup B meningococci and the serotypes 1, 14, 15, and 16 and the subtypes P1.1, P1.4, P1.7, P1.7,1, P1.7,16, P1.9, P1.10, P1.12, P1.15, and P1.16 among serogroup C meningococci were combined. Mantels test was used to test for trends of proportions.[19]

RESULTS

The general results will be presented for the period 1980-1990. In regard to isolates of serogroup B, the findings of the period 1958-1990 are described.

Incidence and serogroup distribution

From 1980 to 1990, isolates of *N. meningitidis* from 3354 patients (1740 males, 1548 females, 66 of unspecified gender; male/female ratio, 1.12) were submitted to the RLBM.

Table 2. Annual number of meningococcal isolates, age-specific and overall incidence rates of meningococcal disease (per 100,000 subjects) in the Netherlands, 1980-1990

Year	No. of isolates	Age-specific incidence (y)						Overall incidence
		0-4	5-9	10-14	15-19	20-24	≥25	
1980	209	11.8	1.7	1.7	1.8	0.2	0.4	1.5
1981	230	11.9	2.9	1.8	1.8	0.7	0.4	1.6
1982	162	8.9	2.0	1.3	1.3	1.1	0.2	1.1
1983	183	10.2	1.5	1.5	1.8	0.3	0.4	1.3
1984	196	11.3	2.5	1.6	1.9	0.7	0.3	1.4
1985	254	13.2	3.9	1.6	3.3	0.9	0.4	1.8
1986	357	16.4	5.5	3.7	4.7	1.4	0.5	2.5
1987	337	16.0	6.6	2.9	4.2	1.4	0.4	2.3
1988	383	17.2	6.8	1.8	6.2	1.4	0.6	2.6
1989	520	23.4	9.1	6.3	6.7	2.1	0.7	3.5
1990	523	22.8	6.8	7.4	8.0	1.7	0.7	3.5

In the 1980s the annual incidence of MD has increased from 1.1 cases per 100,000 persons (162 cases) in 1982 to 3.5 per 100,000 persons (523 cases) in 1990 (Table 2). This increase was caused by a rise in the number of isolates of both serogroup B, which was the predominant serogroup in the Netherlands, and serogroup C (Table 3). The number of serogroup A isolates decreased from 1980 to 1983 and accounted for only 2% of the total number of meningococcal isolates in 1990.

Table 3. Meningococcal disease in the Netherlands, 1980-1990: distribution of serogroups (in absolute numbers) and proportion of strains isolated from blood alone (blood-strains) per year

Variable	Data for indicated year										
	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990
Serogroup											
A	59	48	21	9	6	8	6	8	14	12	11
B	99	117	98	121	147	192	275	263	278	401	385
C	45	60	36	45	40	49	64	56	81	94	115
X	0	1	1	2	0	0	4	0	1	1	1
Y	1	1	1	2	1	2	5	4	2	4	3
Z	0	0	0	1	0	0	0	1	1	1	1
29E	0	0	1	0	0	0	0	0	0	1	0
W-135	5	1	4	3	1	3	3	3	6	3	3
Nongroupable	0	2	0	0	1	0	0	2	0	3	4
Total	209	230	162	183	196	254	357	337	383	520	523
Proportion of blood-strains	8%	10%	12%	15%	13%	12%	13%	17%	14%	19%	21%

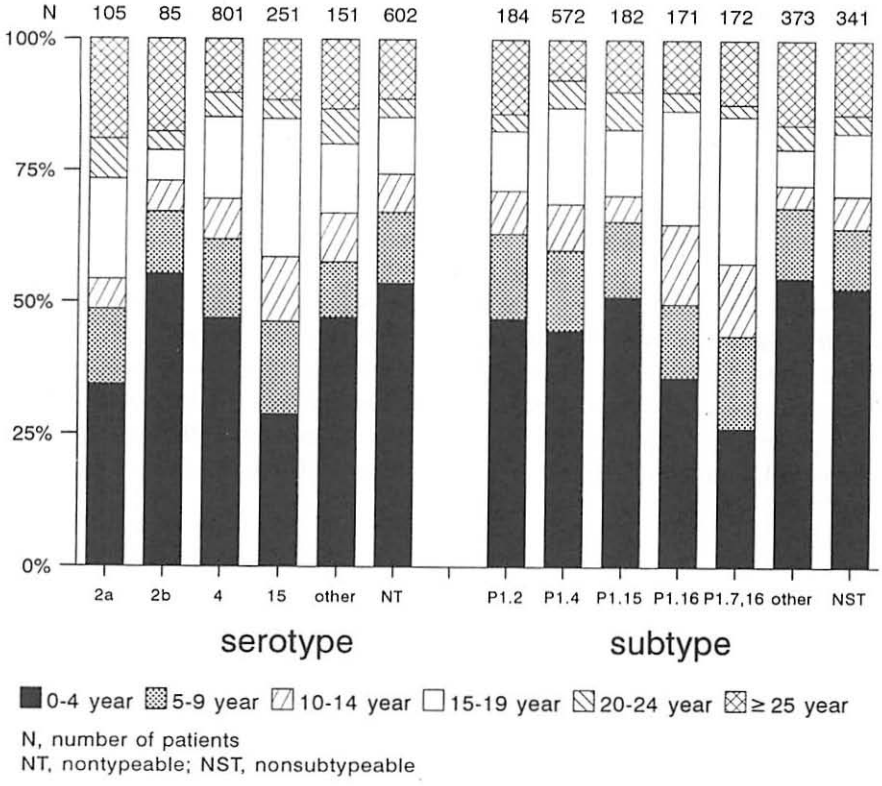

Figure 1. Distribution of age-category of patients with meningococcal disease in the Netherlands, 1980-1990, in relation to main serotypes (left) and subtypes (right) of serogroup B meningococci (n = 1995; age of 14 patients not specified)

Age-distribution

The age-distribution was characteristic of MD. Children aged <5 years were most susceptible, followed by those aged 5-9 and 15-19 years. From 1980 to 1990 the incidence rate in all age-categories increased, but most markedly among individuals between 5 and 24 years old (Table 2). The relative frequency of cases among children aged 0-4 years declined from 52% in 1980 to 41% in 1990, while the proportion of patients in the age-groups 5-9, 10-14, 15-19, and 20-24 increased (χ^2 = 86.2; 50 degrees of freedom [df]: P = .001). The age-distributions differed among the various serogroups (χ^2 = 134.4; 15 df: P < .001). Meningococci of serogroup B affected the highest percentage of children aged 0-4 and 5-9 years (48% and 14%), and serogroup C the highest percentage of children aged 10-14 years and teenagers (15% and 18%). The age-distribution of serogroup A isolates was in between that of serogroups B and C; those isolates designated as belonging to the "other" serogroups affected mainly patients aged ≥25 years (35%) and only 25% of patients 0-4 years old. The shift of

the age-distribution to older age-categories was particularly noted within serogroup B but not within serogroup C (χ^2 = 86.8; 50 df: P = .001 and χ^2 = 43.5; 50 df: P = .729). Within serogroup B a significant association was found between age and serotype (χ^2 = 90.5; 25 df: P < .001) and subtype (χ^2 = 135.5; 30 df: P < .001) (Figure 1). Compared with the serotypes and subtypes that were already present in the Netherlands, the newly-introduced serotypes and subtypes were found relatively more often among patients in the older age-categories. The highest relative frequency of patients aged 0-4 years was associated with serotype 2b isolates, whereas serotype 15 isolates affected the highest percentage of patients aged 5-9, 10-14, and 15-19 years. Compared with those of serotype 2b, serotype 4 isolates were more frequently recovered from patients aged 5-9, 10-14, and 15-19 years. Subtype P1.7,16 isolates affected the highest percentage of patients aged 5-9 and 15-19 years and the lowest percentage of those who were 0-4 years of age, and subtype P1.16 isolates affected the highest percentage of patients aged 10-14 years. Compared with those of subtype P1.2, subtype P1.4 isolates were more often recovered from patients of ages 10-14, 15-19, and 20-24 years.

Source of isolation

The proportion of isolates cultured from blood alone increased significantly from 1980 to 1990 (Mantels test for trend: χ^2 = 37.2; 1 df: P < .001) (Table 3). This proportion differed in the various serogroups (5%, 14%, 18% and 40% for serogroups A, B, C and "other", respectively; χ^2 = 63.6; 3 df: P < .001). The increasing proportion of isolates recovered from blood alone, however, was not due to the changing pattern of serogroups. Among isolates of both the B and C serogroups, the proportion of those from blood alone increased significantly (χ^2 = 19.2; 1 df: P < .001 and χ^2 = 20.5; 1 df: P < .001, respectively), but among those serogroups there was no significant association between source of isolation and serotype (χ^2 = 8.6; 5 df: P = .124 and χ^2 = 4.0; 4 df: P = .412) or subtype (χ^2 = 5.5; 6 df: P = .486 and χ^2 = 4.9; 4 df: P = .299).

Distribution of serotypes and subtypes

Serogroup A. Of the 127 serogroup A isolates obtained in 1980 and 1983 and during 1985-1990, 88 isolates (69%) were of serotype 4, 1 isolate was of serotype 15, and the remaining isolates were nonserotypeable (NT). The most prevalent subtype in serogroup A was P1.10 (66%), followed by P1.16 (22%) and P1.9 (10%). Only 2 isolates were of other subtypes (P1.6 and nonsubtypeable [NST]). With the exception of 4 isolates, only 4 serosubtype combinations were found among serogroup A isolates: 4:P1.10 (74 isolates), NT:P1.10 (10), NT:P1.16 (27) and 4:P1.9 (12). The remaining 4 isolates were identified as 4:P1.6, 4:P1.16, NT:P1.9 and 15:NST. Strain A:4:P1.10 was the most prevalent strain in 1980 (47 isolates; 23% of all isolates). Its frequency dropped in subsequent years. A:NT:P1.10 isolates were found only in 1980, whereas A:NT:P1.16 was present beginning in 1983. A:4:P1.9 isolates appeared after 1986. The frequency of the latter three strains remained low, however.

Table 4. Relative distribution of serotypes and subtypes among serogroup B meningococci recovered from patients with meningococcal disease in the Netherlands between 1958 and 1990 (n = 2357)

	Data for year(s) of recovery											
Variable	1958-61	1964-65	1970	1975	1980	1983	1985	1986	1987	1988	1989	1990
No. of isolates lost in storage	45	23	25	8	3	0	0	1	1	0	0	0
No. of isolates tested	85	81	94	88	96	121	192	274	262	278	401	385
Serotypes (%)												
1	6	6	1	5	6	0	5	0	0	1	1	5
2a	16	9	2	2	2	7	4	4	8	6	6	4
2b	2	15	63	39	31	5	3	6	2	2	2	2
4	4	15	7	11	11	28	35	38	44	47	44	43
14	2	0	0	2	4	6	4	5	5	5	2	3
15	11	5	3	3	1	17	20	20	16	10	9	9
16	2	2	1	3	0	2	3	3	2	0	1	2
4/14	0	0	0	0	0	0	0	0	0	0	0	1
Nonserotypeable	56	48	22	34	44	36	27	25	23	28	35	32
Subtypes (%)												
P1.1	9	0	3	1	1	0	1	0	0	0	1	0
P1.2	22	36	64	39	22	10	9	10	10	8	9	6
P1.4	0	1	1	1	3	10	20	26	24	33	35	41
P1.6	15	12	5	6	7	7	5	1	3	1	2	2
P1.7	2	1	1	1	1	1	2	6	3	3	3	3
P1.7,1	18	14	5	2	3	5	3	2	3	2	1	1
P1.7,16	2	1	0	1	1	7	13	16	11	6	6	6
P1.9	6	1	1	1	3	3	1	3	2	2	2	2
P1.10	0	1	1	1	8	2	2	2	5	5	4	3
P1.12	2	4	1	8	8	7	3	3	1	4	1	3
P1.14	0	0	1	1	1	2	2	3	2	2	3	2
P1.15	0	10	1	9	13	12	16	9	14	8	6	5
P1.16	5	2	0	7	2	10	11	6	10	6	11	9
Other*	2	1	2	0	0	1	1	0	0	0	0	0
Nonsubtypeable	15	15	13	22	26	22	15	13	12	21	16	19

* includes P1.4,16, P1.6,14, P1.6,16, P1.7,14, P1.12,2 (2 isolates), P1.12,4 and P1.12,14

Serogroup B (1958-1990). The distribution of serotypes and subtypes of all available serogroup B isolates obtained during 1958-1961, 1964-1965, 1970, 1975, 1980, 1983, and 1985-1990 (n = 2357) is shown in Table 4. The relative frequency of serotype 2b increased from 2% during 1958-1961 to 15% during 1964-1965, just before the 1966 epidemic, which was caused predominantly by serotype 2b isolates.[16] This serotype was still found to characterize 63% of all serogroup B isolates in 1970, after which its frequency dropped to 2% in 1990. Serotype 2a strains were recovered frequently from 1958 to 1961, but their frequency fell after 1965. Until 1980 the frequency of recovery

of serotype 4 strains varied from 4% to 15%. After 1980 the proportion increased gradually to 47% of all serogroup B isolates in 1988, and it stayed more or less constant until 1990. Two isolates recovered in 1990 were characterized by the combined serotype 4/14. Serotype 15 isolates were found as early as 1960. The prevalence of this serotype during subsequent years was moderate, but increased from 1% in 1980 to 20% during 1985-1986, after which it declined to 9% in 1990.

After 1980 the subtypes P1.16 and P1.7,16, which were frequently linked to serotype 15, followed a time-trend similar to that of the latter. Before 1980 only 4 subtype P1.7,16 isolates were found, but subtype P1.16 isolates were encountered more often. Isolates of subtype P1.2 were responsible for 22% of all serogroup B infections during 1958-1961 and for 64% in 1970. Their frequency fell from 22% in 1980 to 6% in 1990. Only four subtype P1.4 isolates were seen before 1980. The prevalence of this subtype increased sharply from 3% in 1980 to 41% in 1990. Subtype P1.15 isolates were frequently seen during 1964-1965 (10%) and 1975 (9%). From 1980 to 1987, 9%-16% of all serogroup B isolates were of this subtype; its prevalence declined to 5% in 1990. Before 1970, isolates of subtypes P1.6 and P1.7,1 were recovered frequently from patients. Most isolates were of the well-known subtype combinations P1.7,1 and P1.7,16; only eight isolates were of other subtype combinations (P1.4,16, P1.6,14, P1.6,16, P1.7,14, P1.12,2 [2], P1.12,4 and P1.12,14).

Table 5. Association of serotype and subtype among 2357 serogroup B meningococci recovered from patients with meningococcal disease in the Netherlands between 1958 and 1990

	No. of isolates of indicated serotype									
Subtype	1	2a	2b	4	14	15	16	4/14	NT	Total
P1.1	1	0	1	4	1	0	0	0	10	17
P1.2	3	91	146	25	0	1	3	0	59	328
P1.4	1	2	1	447	24	13	0	1	87	576
P1.6	1	1	3	16	6	4	4	0	52	87
P1.7	0	0	0	22	2	10	1	0	29	64
P1.7,1	5	1	0	16	3	0	3	1	43	72
P1.7,16	3	0	0	4	2	153	0	0	14	176
P1.9	0	2	0	4	2	3	0	0	38	49
P1.10	2	2	22	29	2	0	0	0	20	77
P1.12	3	11	3	11	11	2	7	0	24	72
P1.14	1	0	0	27	0	1	1	0	16	46
P1.15	7	1	1	115	3	1	1	0	71	200
P1.16	17	0	0	13	3	64	6	0	81	184
Other*	0	0	1	0	1	2	1	0	3	8
NST	12	19	16	103	23	17	12	0	199	401
Total	56	130	194	836	83	271	39	2	746	2357

* includes subtypes P1.4,16, P1.6,14, P1.6,16, P1.7,14, P1.12,2 (2 isolates), P1.12,4 and P1.12,14
NOTE: NT = nonserotypeable and NST = nonsubtypeable

Over the years, the proportion of NT strains varied irregularly from 22% to 56%, and that of NST strains varied from 12% to 26%.

The most common combinations of serotypes and subtypes among serogroup B meningococci were 2a:P1.2, 2b:P1.2, 4:P1.4, 4:P1.15, 15:P1.16, and 15:P1.7,16 (Table 5). Strain B:2b:P1.2 was isolated once during 1958-1961; its prevalence increased to 15% of all serogroup B isolates during 1964-1965 and to 57% in 1970. Since 1980 it has almost disappeared (17 isolates in 1980 [18%] and 3 [1%] in 1990). The phenotype B:4:P1.4, which was not found before 1980, was encountered twice in 1980. It became the most prevalent phenotype in 1990 (28% of all serogroup B isolates and 21% of all isolates). One isolate of the "Norwegian" epidemic strain B:15:P1.7,16, was found in 1975 and one in 1980. It increased in frequency during subsequent years to 11% of all isolates in 1986, but its frequency declined to 3% in 1990. B:4:P1.15 isolates were found throughout the period of 1958-1990. Their frequency increased moderately during the mid-1980s but decreased after 1987.

Serogroup C. The distribution of serotypes and subtypes of 543 serogroup C meningococci obtained in 1980 and 1983 and during 1985-1990 is shown in Table 6. In the early 1980s serotypes 2a and 2b were scarcely represented, but their relative frequency increased to 48% and 17%, respectively, of all serogroup C isolates in 1990. The prevalence of serotypes 1 and 4 decreased from 15% and 23% to 3% and 9%, respectively. Two isolates were of a combined serotype (4/14 and 4/16). Subtype P1.2 was frequently linked to serotypes 2a and 2b. Its frequency increased from 7% in 1983 to 37% in 1990. Isolates with subtypes P1.4, P1.7 and P1.10 were not found before 1986. The prevalence of subtypes P1.4 and P1.7 remained moderate, but 10% of all serogroup C isolates in 1990 were found to be of subtype P1.10. Isolates of subtypes P1.12 and P1.14 were recovered mainly in the early and mid-1980s. The relative frequency of serogroup C isolates of other subtypes showed only minor fluctuations. One isolate was characterized by 3 subtypes (P1.7,1,15). The proportion of NT isolates varied from 21% to 64%, and that of NST isolates varied from 29% to 43%.

Apart from the combinations 2a:P1.2 (102 isolates), 2a:NST (58), and 4:P1.1 (21), many different serosubtype combinations were found to characterize the 543 serogroup C isolates. Strain C:2a:P1.2 was found only once in 1980, but it became the second most frequently isolated strain in 1990 (6% of all isolates, 28% of all serogroup C isolates).

Serosubtype in relation to ET among serogroup B meningococci, isolated between 1958 and 1986

The ET of 278 serogroup B meningococci recovered between 1958 and 1986 was determined in an earlier survey.[13] Two isolates were lost in storage. Of the remaining 276 isolates, 7 were of phenotype B:4:P1.4 and were all identified as ET-24. With 1 ET-25 isolate (B:4:NST, isolated in 1986) they formed an independent clone lineage (lineage III), which appeared after 1981 in the Netherlands. This lineage contained no other strains, and no P1.4 strains outside this lineage were found.

Table 6. Relative distribution of serotypes and subtypes among serogroup C meningococci recovered from patients with meningococcal disease in the Netherlands between 1980 and 1990 (n = 543)

	Data for year of recovery							
Variable	1980	1983	1985	1986	1987	1988	1989	1990
No. of isolates lost in storage	6	0	0	0	0	0	0	0
No. of isolates tested	39	45	49	64	56	81	94	115
Serotypes (%)								
1	15	4	8	3	7	0	1	3
2a	5	2	20	19	29	35	43	48
2b	0	2	0	5	9	7	7	17
4	21	22	22	13	18	16	11	9
14	5	2	2	2	4	2	1	1
15	3	0	2	3	2	1	2	2
16	0	2	2	2	5	1	1	0
4/14	3	0	0	0	0	0	0	0
4/16	0	0	0	2	0	0	0	0
Nonserotypeable	49	64	43	53	27	37	34	21
Subtypes (%)								
P1.1	3	2	8	5	4	4	5	6
P1.2	10	7	12	17	23	26	38	37
P1.4	0	0	0	2	2	4	2	1
P1.6	15	13	8	13	0	5	3	3
P1.7	0	0	0	0	4	1	2	3
P1.7,1*	8	9	8	13	7	6	6	2
P1.7,16	3	0	0	2	0	4	0	0
P1.9	0	0	2	0	2	0	0	1
P1.10	0	0	0	3	2	1	1	10
P1.12	8	16	8	3	5	1	1	1
P1.14	21	4	6	9	9	0	3	1
P1.15	3	11	6	0	5	4	4	2
P1.16	0	9	6	0	4	1	1	3
Nonsubtypeable	31	29	35	34	34	43	32	32

* includes one isolate of subtype P1.7,1,15, isolated in 1980

Of the 10 B:4:P1.15 isolates, 4 were of the ET-5 in lineage I, which appeared after 1975 in the Netherlands. This lineage is also known as the ET-5 complex. Of the remaining 6 B:4:P1.15 isolates, 5 were of genotypes in lineage X. Those isolates were recovered in 1965 (2), 1975 (1) and 1978 (2). Lineage X contained no other phenotypes. The remaining 18 serotype 4 isolates, which were NST or were of subtypes other than P1.4 or P1.15, were of many different ETs. All 4 isolates of the subtype combination P1.7,16 belonged to the ET-5 complex. Of those 4 isolates, 3 were of serotype 15 (ET-5) and the other was of serotype 14 (ET-3). Of 10 P1.16-isolates, only 2 belonged to the ET-5 complex; the other 8 isolates were of different ETs. All

B:2b:P1.2 isolates (n = 66) belonged to closely related clones of lineage II, strains of which caused the epidemic of 1966 and the hyperendemic wave in 1972, and all B:2a:P1.2 isolates (n = 12) belonged to clones of lineage IX. The remaining 148 isolates were heterogeneous in both serosubtype and ET.

DISCUSSION

Since 1958 the RLBM has been collecting meningococcal isolates recovered from patients with MD in the Netherlands. Since 1972, with the exception of that in 1976, the annual number of meningococcal isolates submitted to the RLBM has exceeded the number of cases reported.[16] In an earlier survey it was calculated that the number of submissions to the RLBM represented >80% of the number of cases of bacterial meningitis in the Netherlands (which was ascertained on the basis of hospital discharge diagnoses), whereas the number of statutory reports represented only 58% of the number of actual cases.[20] Therefore, a high level of representativeness has been reached with the RLBM submission system; however, because the ascertainment rate was lower before 1972, the results obtained with isolates submitted before 1972 should be interpreted with care.

In the last decade the incidence of MD in the Netherlands, calculated from the submissions of meningococcal isolates to the RLBM, has gradually increased from 1.1 cases per 100,000 persons in 1982 to 3.5 cases per 100,000 persons in 1990. During the same period the number of submissions of *Streptococcus pneumoniae* isolates from the CSF of patients with meningitis remained constant, indicating a stable submission pattern.[21] In addition, the number of reports of meningococcal disease in the Netherlands increased from 123 in 1983 to 505 in 1990 (figures obtained from the Department of the Chief Medical Officer of Health). Therefore, the increased incidence of MD in the Netherlands since 1982 is considered a true increase.

A possible explanation for a rise in the number of meningococcal infections is the introduction of a new strain of *N. meningitidis* in a susceptible population, as occurred in the Netherlands during the epidemic of 1966 (strain B:2b:P1.2) and in Norway in 1974 (strain B:15:P1.7,16).[22 23] However, no single strain can be held responsible for the increase of meningococcal infections after 1982 in the Netherlands. Strain B:4:P1.4 was not found before 1980 among the isolates tested. It was isolated twice in 1980 and became the most prevalent strain in 1990 (21% of all isolates). The introduction of this new strain alone can therefore account for only part of the increase. Isolates of strain C:2a:P1.2 were frequently recovered from patients during 1963-1975.[16] Subsequently, their frequency fell to almost zero, but from 1983 onward it increased again; C:2a:P1.2 became the second most prevalent strain in 1990 (6%). Together with strain B:4:P1.15, which seemed to be of a different clone during the 1980s than in the period before, and strain B:15:P1.7,16, newly-introduced isolates accounted for 167 cases (32% of all cases) in the Netherlands in 1990. The occurrence of 100-200 cases more than the usual number of cases that occur in years of

endemicity in the Netherlands (i.e. 150-250 cases per year) remains unexplained. They were not caused by the introduction of new strains but by an increase of a variety of other strains that were already present in the Netherlands.

Several new clones of serogroup B meningococci were found in the Netherlands in the 1980s.[13] From 1983 to 1986 clones of lineage III (ET-24 and ET-25), which appeared after 1981, constituted 15% of the isolates examined. Clones of the ET-5 complex caused several outbreaks of MD all over the world after having caused the Norwegian epidemic.[24] They were first recovered in the Netherlands in 1975. From 1983 to 1986 they constituted 18% of the isolates tested. Thus, from 1983 to 1986 approximately 33% of all infections due to serogroup B strains (i.e. 25% of all isolates) have been caused by isolates of those two new clones. We have demonstrated that lineage III consists almost exclusively of B:4:P1.4 strains. Lineage I contains several phenotypes, of which B:15:P1.7,16 and B:4:P1.15 are the most frequent representatives. The genotype-related findings are therefore in accordance with the phenotype-related findings, i.e. the rise in the number of meningococcal infections in the Netherlands is due not only to new clones of *N. meningitidis*, but also to a variety of clones that have been noted before in the Netherlands.

From 1980 to 1990 there was a shift in the age-distribution of patients with meningococcal disease from younger to older age-categories, particularly with regard to cases due to meningococci of serogroup B. This phenomenon has been observed before and has been explained by a coinciding shift in the serogroup and/or serosubtype distribution.[1 25 26] The appearance of one or more new strains of *N. meningitidis* theoretically would mainly be reflected in patients in the older age-categories, because these patients are relatively more susceptible to new strains than to old strains. The younger patients are equally immune to antigens from both old and new strains of meningococci. Among serogroup B strains a significant association was found between both serotype and subtype and the age of the patient. MD in patients in the older age-categories is more often associated with new serotypes and subtypes than it is in patients aged 0-4 years. A similar association was found between age and the new clone lineages that appeared in the Netherlands after 1980.[13] The shift of the age-distribution to older age categories can therefore be explained by the appearance of new serotypes and subtypes within serogroup B in the Netherlands.

The increase in the proportion of isolates of both serogroups B and C that were cultured from blood alone can be considered a parameter of changed virulence and thus can represent another explanation for the increased incidence of MD. The increased proportion of those isolates, however, could not be attributed to a particular serotype or subtype. It might be possible that the markers used (serotype and subtype) are not appropriate for identifying virulence characteristics. It is also possible that the observed increase in the proportion of isolates recovered from blood only was merely caused by a change in clinical regimen, e.g. more blood-cultures were carried out in the late 1980s than in the early 1980s.

The results of both multilocus enzyme electrophoresis and serosubtyping indicate strain heterogeneity. Together with the gradualness of the increase in incidence of

MD in the Netherlands (in contrast with the abrupt increase that was noted during previous serogroup B-associated epidemics, such as the ones in the Netherlands in 1966 and in Norway in the early 1970s), this heterogeneity suggests the likelihood of other explanations for the increase in addition to the introduction of new clones or serosubtypes. This increase could be due to an overall diminished immunity against meningococci among the Dutch population, a theory that will be subject to further investigation, or an increased circulation of meningococci in general in the Dutch population. In the last decade the Dutch have tended to spend more, shorter holidays in their own country.[27] This increased circulation of inhabitants facilitates the circulation, transmission, and acquisition of the prevailing strains of meningococci. If the increased circulation of meningococci indeed appears to be associated with the increased incidence of MD, then the currently observed high incidence will remain at this level until a vaccine becomes available.

The variety of serosubtypes amongst serogroup B meningococci in the Netherlands, as found in other countries as well, implicates the need for a multivalent OMP vaccine (if such a vaccine proves to be effective on a type-specific basis).[28 29] Currently, the development of a multivalent serogroup B class 1 OMP vaccine, active against all detectable subtypes, is under investigation. In the past 30 years, 20%-25% of the serogroup B isolates in the Netherlands have been NST. Therefore, a multivalent vaccine theoretically would have given protection against 75%-80% of all serogroup B infections in the Netherlands since 1958. Such a vaccine would have to be adapted according to ongoing changes in the distribution of subtypes over time. However, on the basis of the above-mentioned results, these changes would not be needed very often. In this respect, the implementation of a meningococcal vaccine is more complicated than that of the current trivalent polio vaccine but considerably less complicated than the strategy needed for influenza vaccination. Careful surveillance of the distribution of subtypes among serogroup B meningococci will still be needed to ensure that a suitable vaccine is constructed and adapted according to possible shifts in the distribution of subtypes.

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CHAPTER 3

PHENOTYPIC AND GENOTYPIC CHANGES IN A NEW CLONE COMPLEX OF *NEISSERIA MENINGITIDIS* CAUSING DISEASE IN THE NETHERLANDS, 1958-1990

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ABSTRACT

In order to characterize the phenotypic and genotypic changes that occurred in a new clone lineage of *Neisseria meningitidis* (lineage III) in the Netherlands, we determined the electrophoretic type (ET) of 79 serogroup B isolates of serotype 4 and/or subtype P1.4, obtained between 1958 and 1990 from patients with systemic meningococcal disease. 35 previously described isolates were also included in the analysis. Clones of lineage III were not found before 1980 in the Netherlands. After its appearance, the lineage started very homogenously with regard to both the genotype (ET-24) and phenotype (B:4:P1.4). The clone might have had its origin in the Netherlands, as the clone seems to represent a very low proportion of disease in other countries. After 1984, the lineage acquired other clones, which were closely related to ET-24. All subtype P1.4 isolates recovered after 1980 belonged to clones of lineage III, indicating that this subtype was an important marker of this lineage at that time. After 1984 the clones acquired other serotypes (serotypes 14, 15) and subtypes (P1.2, P1.7, P1.12), suggesting the exchange of genetic material between clones. The fairly rapid changes in the serotype and subtype that occurred within the clones in the 1980s implicate the need for regular adaptation of a vaccine, based on the serotype and/or subtype antigens.

INTRODUCTION

Meningococci are classified into serogroups, serotypes and subtypes on the basis of antigenic differences in their capsular polysaccharides, class 2/3 outer membrane proteins (OMPs) and class 1 OMPs, respectively.[1] Studying the distribution of these surface structures has proved to be useful for analysing the spread of meningococcal disease (MD).[2-5] In recent years it has been shown that meningococci have a clonal population structure, and the characterization of the chromosomal genotype of *Neisseria meningitidis* by multilocus enzyme electrophoresis has shown to be an even more powerful tool for studying the epidemiology of MD.[6-8] If closely related meningococcal clones are homogenous with regard to the phenotype, the results of the surface characterization of *N. meningitidis* and genotyping will be similar, and will lead to the same inferences. This was the case with strain B:2b:P1.2 which caused the 1966 epidemic and a hyperendemic wave in 1972 in the Netherlands.[3 4] All B:2b:P1.2 isolates, of which the electrophoretic type (ET) was determined, belonged to a complex of closely related clones (lineage II) and this complex contained only a few other phenotypes.[5 8] However, multilocus enzyme electrophoresis revealed that the clone that caused the hyperendemic wave of 1972 differed from the clone that dominated the 1966 epidemic, and offered, therefore, a further refinement.[8] If, however, a lineage of closely related clones is heterogenous with regard to the phenotype, results obtained by typing methods based on surface antigens may not lead to comparable inferences. An example of this situation is the ET-5 complex. Clones of the ET-5 complex caused several outbreaks of MD all over the world, after having caused the Norwegian epidemic, but many phenotypes were found among isolates of this complex, and epidemics in different countries were often caused by strains with distinct phenotypes (e.g. B:15:P1.7,16, B:4:P1.15 and B:15:P1.3).[2 7]

Since 1958, meningococcal isolates from blood and/or cerebrospinal fluid (CSF) of patients with systemic MD have been submitted for further classification to the Reference Laboratory for Bacterial Meningitis (RLBM) in Amsterdam by almost all clinical microbiological laboratories in the Netherlands. Research on this extensive collection of strains, which currently comprises of approximately 7000 meningococcal isolates, has demonstrated that a new lineage of 2 closely related clones of *N. meningitidis* (lineage III) appeared in the Netherlands around 1980.[8] This lineage was partly responsible for the increase in the incidence of MD in the Netherlands after 1982; it was estimated that approximately 40% of the cases of serogroup B MD in the Netherlands in 1987 were due to clones of this lineage.[8] It has subsequently been demonstrated that this lineage consisted almost exclusively of a new meningococcal phenotype (B:4:P1.4) which was encountered for the first time in the Netherlands around 1980 and became the most prevalent phenotype in 1990 (21% of all isolates).[5] When observing the changing distribution of serotypes and subtypes among serogroup B meningococci in the Netherlands from 1958 to 1990, the subtype P1.4 of the new phenotype deserves special attention. Before 1980 this subtype was rarely encoun-

tered in the Netherlands, but its prevalence increased sharply after 1980, whereas the serotype 4 of this new phenotype had often been identified before 1980.[5]

Clones which are newly-introduced in a certain geographic area will be homogenous with regard to surface characteristics, but clones which have been prevalent in a population for a long period of time might undergo changes in their surface characteristics, due to mutation and/or recombination of genes encoding for surface markers. In that case, the clones become increasingly heterogenous with regard to their phenotype. The noteworthy situation of a homogenous clone lineage in the Netherlands, which was apparently not found before 1980 and increased in frequency during the 1980s, enabled us to study this lineage in more detail. The aim of this study was to characterize the phenotypic and genotypic changes that occurred in this new clone lineage after its appearance in the Netherlands.

MATERIALS AND METHODS

Bacterial isolates

The study base consisted of all previously described serogroup B meningococcal isolates, which were of serotype 4 and/or subtype P1.4, obtained during the periods 1958-1961, 1964-1965, 1970, 1975, 1980 and 1983-1990 (n = 1020).[5] A selected sample of 79 isolates was drawn from this study base for the determination of the ET. This sample included the following isolates: all subtype P1.4 isolates (n = 7) and a random sample of 18 serotype 4 isolates, collected ≤1980, and a random, stratified sample of B:4:P1.4 (n = 13), B:4:non-P1.4 (n = 21) and B:non-4:P1.4 isolates (n = 20), obtained in 1984-1986 and 1990. In addition, we included in the analysis 22 serogroup B:serotype 4 and/or subtype P1.4 isolates with a known ET,[8] obtained in the same periods as the above-mentioned 79 isolates.

In the analysis of qualitative phenotypic changes in clones of lineage III, we added to the lineage III isolates found in this study (n = 47) 3 previously described lineage III isolates obtained in 1982 and 1983[8] and 10 lineage III isolates obtained in 1987, of which the ET had already been determined.

Presence of a class 1 OMP

All nonsubtypeable (NST) isolates, of which the ET was determined (n = 15), were screened for the presence of a class 1 OMP by means of a 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell suspensions. The proteins were stained with Coomassie brilliant blue.

Electrophoresis of enzymes

Methods of starch-gel electrophoresis and selective enzyme staining were similar to those described by Selander, *et al.*[9] Multilocus enzyme electrophoresis of the isolates and analysis of the results were performed as described.[8] The electrophoretic types identified were compared with those distinguished among the 278 previously analysed

meningococcal isolates from the Netherlands, and designated accordingly.[8] New ETs were designated by adding a figure to the most closely related ET of the former set of strains.

RESULTS

Evolution of phenotypes

Figure 1 shows the relative distribution of serotype 4, subtype P1.4 and phenotype 4:P1.4 isolates among serogroup B meningococci in the Netherlands from 1958 to 1990. Serotype 4 was found in combination with various subtypes among serogroup B isolates throughout the study-period. Its prevalence, which varied from 4-15% during the period 1958-1980, increased to 43% in 1990.[5] This increase was mainly due to the increase of B:4:P1.4 strains in the last decade. The phenotype B:4:P1.4 was first encountered in 1980 (2 isolates). In subsequent years its relative frequency gradually increased to 28% of all serogroup B isolates in 1990.[5] Before 1980, only 4 subtype P1.4 isolates were found: 2 isolates in combination with serotype 15, isolated in 1965 and 1970, and 2 nonserotypeable (NT) strains, isolated in 1965 and 1975, respectively. Together with the two above-mentioned B:4:P1.4 isolates, only one other P1.4 strain

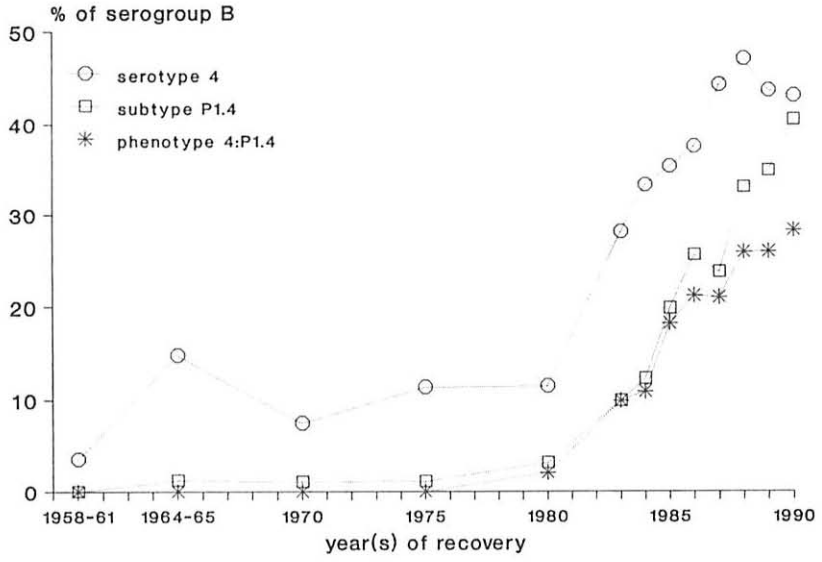

Figure 1. Relative distribution of serotype 4, subtype P1.4 and phenotype 4:P1.4 among serogroup B meningococci in the Netherlands, 1958-1990

was found in 1980 (B:NT:P1.4). After 1980 the proportion of P1.4 isolates increased to 41% of all serogroup B isolates in 1990.[5] From 1980 to 1985 subtype P1.4 was mainly found in combination with serotype 4, but thereafter an increasing number of NT strains and isolates with serotype 14 and 15 were encountered among P1.4 isolates (Table 1).

Table 1. Distribution of serotypes among 595 serogroup B subtype P1.4 meningococcal isolates, obtained in the Netherlands between 1958 and 1990

	No. of isolates in indicated year(s)										
Serotype	<1980	1980	1983	1984	1985	1986	1987	1988	1989	1990	Total
4	-	2	12	16	35	58	55	72	104	109	463
14	-	-	-	-	-	4	2	6	4	8	24
15	2	-	-	-	-	2	2	2	4	1	13
other*	-	-	-	-	-	2	-	-	2	1	5
nonserotypeable	2	1	-	2	3	4	3	12	26	37	90
Total	4	3	12	18	38	70	62	92	140	156	595
Total number of serogroup B isolates	348	96	121	147	192	274	262	278	401	385	2504

* includes 2 B:2a:P1.4 isolates (1986), 1 B:2b:P1.4 (1989), 1 B:1:P1.4 (1989) and 1 B:4/14:P1.4 (1990)

Distribution of clone lineage by phenotype

Six very closely related clones (ET-24, ET-24.1 through ET-24.4, and ET-25) which differed at ≤2 alleles from each other, were identified as belonging to lineage III. The distribution of clone lineage III and non-lineage III over the various phenotypes of the 101 meningococcal isolates is shown in Table 2. All 19 B:4:P1.4 isolates were of lineage III: 15 were ET-24, 1 ET-24.2 (isolated in 1984), 1 ET-24.3 (1990) and 2 ET-24.4 (1986 and 1990). The 8 B:14:P1.4 isolates, which were all recovered after 1985, also belonged to this lineage, but only 1 isolate shared an ET (ET-24) with the B:4:P1.4 strains. The B:4/14:P1.4 isolate was also ET-24. Of the remaining 16 subtype P1.4 isolates, 11 strains which were isolated after 1980 (3 B:15:P1.4 and 8 B:NT:P1.4 isolates) were of ETs in lineage III, but the ETs of the 2 B:15:P1.4 and 3 B:NT:P1.4 isolates recovered in the period 1958-1980 were not related to those of lineage III. The same applies to the 5 B:4:NST isolates which were recovered in 1959 (1 isolate), 1975 (3) and 1980 (1). Of the remaining 10 B:4:NST isolates which were found after 1980, 5 had ETs which were unrelated to those of lineage III, 4 were ET-24 and 1 was ET-25. All 5 B:4:NST isolates of lineage III expressed a class 1 OMP, as determined by SDS-PAGE, but 2 of the 10 non-lineage III B:4:NST isolates had no class 1 OMP. The ETs of the majority of the 42 serotype 4 isolates with subtypes

Table 2. Distribution of clone lineage III among 101 serogroup B meningococcal isolates, obtained in the Netherlands between 1958 and 1990, according to phenotype and year(s) of recovery

	No. of isolates in indicated year(s)											
	Total no. of isolates	<1980		1980		1984-86		1990		Total	
Phenotype*	tested	Lineage: III	other	Lineage: III	other	Lineage: III	other	Lineage: III	other	Lineage: III	other
B:4:P1.4	19	-	-	2	-	11	-	6	-	19	-
B:14:P1.4	9	-	-	-	-	4	-	5†	-	9	-
B:15:P1.4	5	-	2	-	-	2	-	1	-	3	2
B:NT:P1.4	11	-	2	-	1	4	-	4	-	8	3
B:4:NST	13	-	3	-	1	2	3	3	1	5	8
B:4:P1.-‡	2	-	1	-	-	-	1	-	-	-	2
B:4:other**	42	-	18	-	4	1	12	2	5	3	39
Total	101	-	26	2	6	24	16	21	6	47	54

* NT = nonserotypeable; NST = nonsubtypeable

† includes one B:4/14:P1.4 isolate

‡ no class 1 outer membrane protein present

** subtype other than P1.4 or NST; in lineage III: B:4:P1.2 (1984), B:4:P1.7 (1990) and B:4:P1.12 (1990)

other than P1.4 differed from those of lineage III, with the exception of 3 isolates which were in lineage III (ET-24). These 3 isolates were all recovered after 1980 and expressed the subtypes P1.2 (recovered in 1985), P1.7 (1990) and P1.12 (1990), respectively.

Genotypic and phenotypic changes in lineage III

The first representatives of lineage III were encountered in 1980. During the period 1980-1983 only ET-24 was found in this lineage, but from 1984 onwards other ETs also appeared. The distribution of phenotypes over the various ETs of isolates of lineage III (n = 60) is shown in Table 3. From 1980 to 1983 ET-24 was solely represented by isolates of the phenotype B:4:P1.4, but subsequently isolates of the serotypes 14, 4/14, NT isolates, isolates of the subtypes P1.2, P1.7, P1.12 and NST isolates appeared in this clone. The other ETs of lineage III were each represented by 1 to 8 isolates of various phenotypes, including B:4:P1.4. All isolates of these clones were of subtype P1.4, with the exception of one ET-25 isolate recovered in 1986, which was NST. This isolate, as well as the other 4 NST isolates of clone lineage III, expressed a class 1 OMP, indicating the presence of at least one other, but yet unknown subtype among clones of lineage III.

Table 3. Distribution of phenotypes according to electrophoretic type (ET) among 60 serogroup B meningococcal isolates of clone lineage III in the Netherlands, 1980-1990

	Phenotype* in indicated year(s) (no. of isolates, if > 1)		
ET no.	1980-83	1984-87	1990
ET-24	4:P1.4 (5)	4:P1.4 (16) NT:P1.4 (4) 4:NST 4:P1.2 4:P1.7	4:P1.4 (4) NT:P1.4 (2) 4:NST (3) 4:P1.7 4:P1.12 4/14:P1.4 14:P1.4
ET-24.1		15:P1.4	15:P1.4 14:P1.4
ET-24.2		4:P1.4 (2)	NT:P1.4
ET-24.3			4:P1.4
ET-24.4		4:P1.4 (2) 15:P1.4	4:P1.4
ET-25		14:P1.4 (4) 4:NST	14:P1.4 (2) NT:P1.4

* NT = nonserotypeable; NST = nonsubtypeable

DISCUSSION

The aim of this study was to describe the genotypic and phenotypic changes that occurred in a new clone lineage of *N. meningitidis* after its appearance in the Netherlands. We therefore determined the ETs of serogroup B isolates of serotype 4 and/or subtype P1.4. The choice of this serotype and subtype was based on the results of two previous reports, in which it was demonstrated that a new meningococcal clone lineage appeared in the Netherlands in 1980, which was mainly characterized by the phenotype 4:P1.4.[5 8] By using this strategy, we expected to enhance the chances of achieving our goals. However, it can not be excluded that representatives of the new lineage in the period before 1980 have been overlooked, or that a change of the phenotypes of the clones to other phenotypes than the ones under study has not been detected.

In the previously described survey of randomly selected meningococcal isolates,[8] and in our selected sample, no clones of lineage III were found in the Netherlands before 1980. After its appearance in 1980, this lineage was represented by one single clone (ET-24) and remained homogenous until 1984. From 1980 to 1983 this clone was represented by one single phenotype (B:4:P1.4). The absence of isolates of

lineage III before 1980, and the limited phenotype and genotype diversity of the lineage during the first years after its appearance, suggest an immigration of this new clone of *N. meningitidis* into the Netherlands around 1980. However, we can not exclude the possibility that the clone was already present in the Netherlands, either among patients or carriers, and had acquired a virulence factor. Moreover, we only analysed serogroup B strains, and the clone could have been present among isolates of the other serogroups. Clones of lineage III were not found in other countries until the late 1980s: a limited number of these clones were found in the United Kingdom in 1987 and 1988, and in Greece and Austria in 1990 (Caugant *et al.*; unpublished observations). In the course of the Norwegian vaccination trial, one B:4:P1.4 isolate was identified,[10] and 2 additional isolates were found in the 1990s (Caugant *et al.*; unpublished observations). In all the above-mentioned countries, clones of lineage III seem to have caused only a very low proportion of the disease. Therefore, it might well be possible that the clone originated in the Netherlands.

Of the strains from which the ET was determined, all B:4:P1.4 isolates and all other P1.4 isolates recovered after 1980 belonged to lineage III, indicating that the subtype P1.4 in this period is an important marker of this lineage. In the late 1980s, an increasing number of P1.4 strains with serotypes other than serotype 4 were found, in particular serotype 14 and NT strains. In addition, new clones seem to have derived from the original ET-24 from 1984 onwards. These are strong indications for the fairly rapid divergence of both the genotype and phenotype of a newly-introduced clone, which occurred in a relatively short period of time.

In the early 1980s, clone ET-24 consisted only of the phenotype B:4:P1.4, but from 1984 onwards the clone acquired a number of other phenotypes. ET-25 predominantly comprised the phenotype B:14:P1.4, but B:4:NST and B:NT:P1.4 were also found. These results give circumstantial evidence for the exchange of (parts of) the porB and porA genes, which encode for the serotype and subtype, respectively, between other clones and clones of lineage III. Given the phenotypes which we have found, exchange of porB genes seems to occur more frequently than exchange of porA genes.

In conclusion, a new lineage of clones of *N. meningitidis* appeared in the Netherlands around 1980, commencing very homogenously with regard to both genotype and phenotype. Until 1984 this lineage remained rather stable, but subsequently other clones appeared which were closely related to the first clone. Phenotypic changes also occurred within these clones, indicating the exchange of genetic material between clones. The results of genotyping and phenotyping were complementary and provided deeper insight into the evolution of meningococcal strains. By studying the closely related clones of lineage III in the Netherlands, it was found that changes in the serotype and subtype occur over a period of several years, indicating that a vaccine based on the serotype and/or subtype antigens will need to be adapted from time to time in accordance with the changing situation.

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CHAPTER 4

LIPOOLIGOSACCHARIDE IMMUNOTYPING OF *NEISSERIA MENINGITIDIS* BY A WHOLE-CELL ELISA USING MONOCLONAL ANTIBODIES

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ABSTRACT

In order to assess the applicability of the whole-cell ELISA (WCE) with monoclonal antibodies for lipooligosaccharide (LOS) immunotyping of *Neisseria meningitidis*, we analysed 675 meningococcal isolates obtained in 1989 and 1990 in the Netherlands, and 57 isolates collected in 1974, of which the immunotype was determined previously by microprecipitation. Despite the lack of specific monoclonal antibodies for L2 and L4, we were able to develop an algorithm for the assignment of immunotypes on the basis of the reaction patterns of the reference strains and these isolates to a combination of 14 monoclonal antibodies. The immunotypes found by WCE were in accordance with those obtained by microprecipitation. In addition, the results from the WCE were reproducible. The distribution of immunotypes among isolates of the various serogroups in the Netherlands in 1989-1990 is presented. Based on the reaction patterns of the isolates, 2 main categories of related immunotypes could be distinguished among isolates of the serogroups B and C: L2/L4 and L3/L1/L8. Some isolates of the latter category were of 1 immunotype, but many isolates expressed, either strongly or weakly, 1 or 2 additional immunotypes, indicating that the differences in this category are quantitative rather than qualitative, which is conceivable in view of the chemical structure of these immunotypes. The results of this study have demonstrated that the WCE method for LOS immunotyping is easily applicable and provides the possibility of better definition of test strains for *in vitro* bactericidal assays and pathogenesis research.

INTRODUCTION

In 1985, a scheme was proposed for the characterization of *Neisseria meningitidis*.[7] It consisted of a combination of serogroup, serotype, subtype and lipopolysaccharide (LPS) serotype. The name of the latter was changed to lipooligosaccharide (LOS) immunotype (IT) after it had been demonstrated that the oligosaccharide structure of the LPS of the meningococcal outer membrane determined the LPS serotype.[24] Serogrouping, serotyping and subtyping has been proved useful for studying the epidemiology of meningococcal disease, but LOS immunotyping is not regularly incorporated in epidemiological studies concerning *N. meningitidis*. LOS immunotyping was hindered by the complexity of the methods involved,[15 27] but in recent years an increasing number of monoclonal antibodies (moabs) have been developed, facilitating immunotyping.[4 11 20] However, there is still no moab available for every IT. Moreover, the interpretation of immunotyping results is difficult, because meningococci often express several ITs, and the expression of ITs may be influenced by growth conditions.[13 15 16 25 27 28] At present, 11 different LOS ITs are recognized: L1 through L11, of which L3, L7 and L9 are immunochemically closely related.[12 15 27 28] The chemical structure of the terminal structures of L1 through L6 and L8 has been elucidated.[5 6 8 9 14 21] Recently, 2 new LOS ITs (L12 and L13) were found among serogroup A meningococci.[3] Their true existence, however, has not yet been established, because it is not clear whether they indeed represent new LOS structures. L9 through L11 are predominantly found among serogroup A meningococci, and L2 through L4 among the serogroups B and C.[3 11 15 19 27 28] Knowledge of the distribution of LOS ITs among serogroup B meningococci might become of special interest, because the LOS is a potential vaccine candidate.[18]

The aim of this study was to evaluate the applicability of the method of whole-cell ELISA (WCE) for LOS immunotyping using monoclonal antibodies. Despite the lack of specific monoclonal antibodies for every IT, we were able to develop an algorithm for the assignment of immunotypes, based on the WCE results. We determined by WCE the IT of 57 patient strains collected in 1974, and compared the results with the previously described results of microprecipitation.[15] We assessed the reproducibility of the WCE method by comparing the immunotyping results of 2 separate cultures of 116 isolates. In addition, we report on the distribution of LOS ITs among 563 consecutive patient strains, obtained in the Netherlands in 1989-1990, and their association with the serogroups, serotypes and subtypes.

MATERIALS AND METHODS

Bacterial isolates

Since 1958, isolates of *N. meningitidis* recovered in the Netherlands from the blood and/or cerebrospinal fluid (CSF) of patients with meningococcal disease, have been sent on chocolate-agar slants to the Netherlands Reference Laboratory for Bacterial

Meningitis in Amsterdam by regional laboratories. Upon arrival, the isolates are serogrouped and stored at -70°C on glass beads as suspensions in 15% glycerol broth.[23]

This study concerned the meningococcal isolates of 563 consecutive patients with meningococcal disease, collected in the period from 1989 to 1990. Isolates from both the blood and CSF were obtained from 112 patients, giving a total number of 675 isolates for the analysis. Also included were 57 patient isolates, collected in 1974, of which the IT was determined previously by microprecipitation.[15] The following reference strains were used: 126E (L1), 35E (L2), 6275 (L3), 89I (L4), M981 (L5), M992 (L6), 6155 (L7), M978 (L8), 120M (L9), 7880 (L10), and 7889 (L11).

The isolates were cultured overnight on chocolate agar plates at 37°C in a humid atmosphere containing 5% CO_2. The bacteria were scraped from the plates with cotton swabs, resuspended in phosphate-buffered saline of pH 7.2 and heat inactivated for 30 min at 56°C. The concentration of the suspensions was adjusted to an optical density (OD) of approximately 0.100, measured in a standard 1 cm cuvette at 620 nm with a Titertek multiscan (Flow Laboratories). The suspensions were stored at 4°C until further processing.

Serotyping and subtyping

Serotyping and subtyping were performed by a WCE, as described previously.[1] The panel of moabs included moabs against serotypes 1, 2a, 2b, 4, 14, 15 and 16, and subtypes P1.1, P1.2, P1.4, P1.5, P1.6, P1.7, P1.9, P1.10, P1.12, P1.14, P1.15 and P1.16. The moab against subtype P1.5 (Mn22A9.19) has been developed recently, and was defined to be specific for the class 1 outer membrane protein (OMP) by means of immunoblotting analysis.

LOS immunotyping

LOS immunotyping was done by means of a WCE, as previously described,[1] using moabs against LOS of *N. meningitidis*. The moabs were produced and tested for specificity as described elsewhere.[2 4 11 20 29] In general, outer membrane complexes were used as immunizing and detecting antigens in order to develop OMP specific moabs.[2 29] Over the years, a number of LOS moabs have been isolated during the development of OMP moabs. The antibodies were characterized by immunoblotting and WCE, and the most useful ones were used in this study. Moabs Mn4D1B3 and Mn4A8B2 were made after immunization with oligosaccharide-tetanus toxoid conjugates.[26] The panel of moabs is shown in Table 1. Moabs 17-1-L1, 9-2-L3,7,9, 2-1-L8 and 14-1-L10 were from the laboratory of WDZ. All moabs are of the IgG class, except Mn4D1B3, which is IgM. For the latter moab, anti-mouse immunoglobulin was used as a conjugate in the WCE instead of protein-A-peroxidase. The ELISA results were read with a Titertek multiscan (Flow Laboratories) at 450 nm. The OD cut-off values for a positive reaction in the WCE were dependent on the moab, the non-specific background of negative controls and the intensity of the reaction with positive controls. The reaction pattern of each isolate to the 14 moabs was determined on the basis of the individual OD cut-off values of the moabs.

In order to assess the comparability of the WCE method with the microprecipitation method for immunotyping, the WCE results of the 57 isolates collected in 1974 were compared with the results obtained by microprecipitation, as previously described.[15]

To assess the reproducibility of the WCE method for immunotyping, the 57 isolates obtained in 1974, as well as 59 of the isolates obtained in 1989-1990, were cultured and immunotyped twice, and both ITs of each of the 116 pairs were compared.

RESULTS

Reference strains

Table 1 shows the reactions of the 14 moabs with the 11 LOS reference strains in the WCE. Five moabs reacted exclusively with one reference strain: moabs Mn14F21-11 and 17-1-L1 with 126E (L1), 14-1-L10 with 7880 (L10), Mn3A8C with M981 (L5), and Mn4C1B with M992 (L6). The other 9 moabs reacted with two or more reference strains, indicating the presence of shared or additional LOS epitopes on some reference strains.

Table 1. Monoclonal antibodies used for LOS immunotyping of *Neisseria meningitidis* and results of immunotyping of 11 LOS reference strains in a whole-cell ELISA (optical densities)

	Reference strain (immunotype)										
Monoclonal antibody	126E (L1)	35E (L2)	6275 (L3)	89I (L4)	M981 (L5)	M992 (L6)	6155 (L7)	M978 (L8)	120M (L9)	7880 (L10)	7889 (L11)
Mn14F21-11	1.8	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1
17-1-L1	2.4	.2	.2	.2	.2	.2	.2	.3	.3	.1	.2
Mn14F20-11	.6	.1	.1	.1	.1	.1	.2	2.5	.2	.5	1.7
Mn13H21	1.3	.2	.4	.1	.1	.1	.6	2.2	1.4	1.8	1.9
2-1-L8	1.7	.3	.6	.2	.2	.2	.6	2.4	1.1	1.4	1.7
14-1-L10	.1	.1	.1	.1	.1	.1	.1	.1	.1	1.9	.1
Mn11A11G	.1	.2	.8	.1	.1	.1	.1	.1	.3	.2	.6
Mn15A17F12	.1	.8	1.7	.3	.8	.1	1.8	.6	2.1	2.0	.1
9-2-L3,7,9	.1	1.0	2.3	.3	.7	.1	2.4	2.2	2.4	.2	.1
Mn15A8-1	.2	2.0	2.3	1.7	2.1	.2	2.1	1.9	2.3	.2	.1
Mn4D1B3	.2	.5	1.6	.2	.2	.2	1.7	1.3	1.6	.2	.2
Mn4A8B2	.1	.1	1.5	.1	.1	.1	1.6	.3	1.6	.1	.1
Mn3A8C	.1	.1	.1	.1	1.9	.1	.1	.1	.1	.1	.1
Mn4C1B	.1	.2	.1	.3	.1	1.4	.1	.1	.4	.1	.1

Table 2. Algorithm for the assignment of LOS immunotypes of *Neisseria meningitidis* as determined in a whole-cell ELISA with a panel of 14 monoclonal antibodies*

Monoclonal antibody	Immunotype								
	L1	L2†	L3	L4	L5	L6	L8‡	L10	L11
Mn14F21-11 and/or 17-1-L1	+								
Mn14F20-11		-		-			+		
Mn13H21 and/or 2-1-L8		-		-					
14-1-L10								+	
Mn11A11G		-		-					+
Mn15A17F12		+		-					
9-2-L3,7,9		+		-					
Mn15A8-1		+		+					
Mn4D1B3		-		-					
Mn4A8B2		-	+	-					-
Mn3A8C		-		-	+				
Mn4C1B						+			

* + = positive reaction; - = negative reaction

† L2 is assigned in case of a positive reaction with Mn15A8-1 and either one or both of the moabs Mn15A17F12 and 9-2-L3,7,9

‡ in case of a positive reaction with both Mn14F20-11 and Mn11A11G, but with a negative reaction for Mn4A8B2, L11 is assigned

Assignment of LOS immunotypes

On the basis of the observed reaction patterns of the 675 patient isolates of 1989-1990 with the 14 moabs, and the knowledge of the reactions of the various moabs with the reference strains, an algorithm for the assignment of ITs was developed (Table 2). The following ITs are discerned: L1, L2, L3, L4, L5, L6, L8, L10 and L11. L7 and L9 could not be separated from L3 by our set of moabs, and were considered to be L3. An isolate is labelled L3 if it reacts with moab Mn4A8B2. Almost all L3 isolates also reacted with moabs Mn4D1B3, Mn15A17F12, 9-2-L3,7,9 and Mn15A8-1, and many with Mn11A11G. L8 is assigned to an isolate if it reacts with moab Mn14F20-11, but there is one exception: if an isolate reacts with both Mn14F20-11 and Mn11A11G, and not with Mn4A8B2, it is labelled L11. The majority of the L8 isolates also reacted with moabs 2-1-L8 and Mn13H21. Because no immunotype-specific moabs for L2 and L4 exist, the assignment of these immunotypes is based on the reaction pattern of an isolate with several moabs. L2 is assigned if a positive reaction is found with moab Mn15A8-1 in combination with either one or both of the moabs Mn15A17F12 and 9-2-L3,7,9, but with negative reactions for moabs Mn4A8B2, Mn4D1B3, Mn11A11G, Mn3A8C, Mn13H21, 2-1-L8 and Mn14F20-11. If the latter reaction pattern is observed in combination with negative reactions for both moabs Mn15A17F12 and 9-2-L3,7,9, L4 is assumed to be present. For the assignment of L2 and L4, the exclusion of an additional positive reaction with Mn14F20-11 is made,

under the assumption that the combinations L2,8 and L4,8 do not exist (see Discussion), in which case L8 is assigned. The assignment of immunotypes L1, L5, L6 and L10 is based on a positive reaction with the specific moabs described above. All L10 isolates also reacted with Mn15A17F12. Combinations of immunotypes, such as L1,8, L3,1, L3,1,8 and L3,8, are also discernible. A positive reaction with the combination Mn15A8-1 (with or without Mn15A17F12 or 9-2-L3,7,9) on the one hand, and Mn13H21, 2-1-L8 or Mn4D1B3 on the other, is considered non-interpretable in the absence of a positive reaction with one of the other moabs. Isolates that failed to react with any moab were labelled nontypeable (NT).

Immunotypes and moab reaction patterns

Table 3 shows examples of the reaction patterns of isolates of the various immunotypes and immunotype combinations. For the ITs L2 through L6, isolates with a "pure" reaction pattern were found. However, all L1 and L10 and many L3 strains showed some reaction with the L8 group of moabs (Mn13H21 and 2-1-L8), and many L8 isolates with the L3 group (Mn4D1B3, Mn15A17F12, 9-2-L3,7,9, Mn15A8-1), indicating that the isolates under consideration partly express other ITs.

Table 3. Examples of reaction patterns of isolates of various immunotypes and immunotype combinations to a panel of 14 monoclonal antibodies in a whole-cell ELISA

		Monoclonal antibody											
Strain ID	Immunotype	Mn4F20-11 Mn14F21-11 and/or 17-1-L1		Mn13H21 and/or 2-1-L8		14-1-L10 Mn11A11G	Mn15A17F12 9-2-L3,7,9		Mn15A8-1 Mn4D1B3		Mn4A8B2 Mn3A8C		Mn4C1B
892633	1	+	w	+									
900997	1,8	+	+	+									
901005	1,8	+	+	+			w	+	+				
891357	2						+	+	+				
892385	3					+	+	+	+	+	+		
900755	3			w			+	+	+	+	+		
900820	3			+		+	+	+	+	+	+		
892463	3,1	+					+	+	+	+	+		
891177	3,1	+		w			+	+	+	+	+		
900747	3,1,8	+	+	+			+	+	+	+	+		
891021	3,8		+	+			+	+	+	+	+		
892214	4								+				
M981	5							w	+			+	
M992	6												+
900714	8		+	+				w	w				
891165	8		+	+			+	+	+	w	w		
900018	8,10		+	+	+		+						
900100	10			w	+	w	+						
892411	10,11		+	+	+	+	+						
7889	11		+	+		+							

NOTE: + = positive reaction; w = weak positive reaction

The reference strains 126E (L1), 6275 (L3), M978 (L8) and 7880 (L10) did not show "pure" patterns (Table 1). It was impossible to assign an IT to 12 of the 675 isolates, because of an non-interpretable reaction pattern. Only 5 isolates were NT. L5 and L6 were found only among the reference strains.

Comparison of immunotyping by whole-cell ELISA and microprecipitation

The immunotypes obtained in the WCE, using monoclonal antibodies, agreed well with those previously obtained by microprecipitation using polyclonal antibodies[15] (Table 4). Of the 57 isolates tested, 44 (77%) showed identical immunotypes in both methods. For 3 isolates (5%) the results of the two methods disagreed completely. One isolate was L2 by WCE and NT by microprecipitation. A second culture of this isolate was again found to be L2 by WCE. One isolate, which was L2 by microprecipitation, was repeatedly L8 by WCE. The third isolate, which was L3 by microprecipitation, was assigned L8 by WCE, whereas a second culture of this isolate was NT by WCE, although it reacted weakly with the L8 group of moabs. For 10 isolates (18%) minor differences were observed. Two L8 isolates and 1 L3,8 by WCE were typed L1,8 and L3,1,8, respectively, by microprecipitation. However, it was not possible to discriminate between L8 and L1,8 or L3,8 and L3,1,8 by microprecipitation using polyclonal antibodies.

Table 4. Comparison of LOS immunotypes of 57 meningococcal isolates as obtained by microprecipitation using polyclonal antibodies (reference 15) and whole-cell ELISA using monoclonal antibodies

Immunotype determined by:		
Microprecipitation	Whole-cell ELISA	No. of isolates
L1	L1	1
L1	L1,8	1
L1,8	L1,8	3
L1,8	L8	2
L2	L2	16
L2	L2,1	1
L2	L8	1
L2,11	L2	1
L3	L3	19
L3	L3,8	4
L3	L8	1
L3,1,8	L3,1,8	2
L3,1,8	L3,8	1
L6	L6	1
L10	L10	1
Nontypeable	L2	1
Nontypeable	Nontypeable	1

Reproducibility of the whole-cell ELISA method for LOS-immunotyping

The results of immunotyping by WCE of 2 separate cultures of 116 isolates are shown in Table 5. Both immunotypes were identical in 104 pairs (90%), 7 pairs (6%) showed minor differences, and in 5 pairs (4%) a major difference was found. The major difference of 1 pair (L8 and NT, respectively) pertained to an isolate that also showed a different result by comparing WCE with microprecipitation. The second culture of this isolate reacted weakly with Mn14F20-11 (L8). In 3 discordant pairs, one of the isolates was typed L2 and the other showed an non-interpretable result. In the last discordant pair the first isolate was L4 and the second L2. In 5 of the 7 pairs with a minor difference, the difference was due to a weak reaction with a moab essential for that specific IT assignment, and in two instances there seemed to be a true additional IT in one of both cultures of the same isolate.

Table 5. Comparison of LOS immunotypes of 116 meningococcal isolates of 2 separate cultures obtained by whole-cell ELISA

Immunotype determined in:		
Culture 1	Culture 2	No. of isolates
L1	L1	2
L1,8	L1,8	15
L2	L2	28
L2	Non-interpretable	2
L2,1	L2	1
L3	L3	30
L3	L3,6	1
L3,1	L3,1	2
L3,1	L3,1,8	1
L3,1,8	L3,1,8	4
L3,1,8	L3,8	1
L3,8	L3	2
L3,8	L3,8	9
L4	L2	1
L6	L6	1
L8	L8	9
L8	L1,8	1
L8	Nontypeable	1
L8,10	L8,10	1
L10	L10	1
Nontypeable	Nontypeable	1
Non-interpretable	L2	1
Non-interpretable	Non-interpretable	1*

* both cultures showed identical moab reaction patterns

Distribution of LOS immunotypes among meningococcal isolates obtained in 1989-1990

Isolates recovered from both CSF and blood were obtained from 112 of the 563 patients. In the majority of the 112 paired strains, both immunotypes were identical. Only 16 pairs (14%) showed a difference. In 5 of these 16 pairs the differences were striking. One pair showed totally discordant IT results (L2 and L1,8), and these 2 isolates did not have a positive reaction in common with any of the 14 moabs. In 1 pair the blood isolate was L2 and the CSF isolate L4. In 3 instances, 1 isolate of each pair reacted strongly with Mn4A8B2 (L3), while the other did not (2 isolates), or only weakly (1 isolate). In the 11 pairs with only minor differences, the differences pertained to the presence or absence of an additional L8, but in 10 pairs the non-L8 isolate reacted weakly with Mn14F20-11 (L8), indicating the presence, though weakly expressed, of an additional L8 IT. In the analyses of the next sections, only 1 strain of each patient is included. The L2-L4 pair is considered to be L2, and the other 14 different pairs are considered to be L3,8. The completely discordant pair is omitted, leaving 562 isolates for the further analyses.

Table 6 shows the distribution of LOS immunotypes by serogroup among 562 meningococci obtained in 1989-1990 from patients with meningococcal disease. The most prevalent immunotypes are L3 (37%), L3,8 (22%), L2 (14%), L1,8 (9%) and L4 (6%). The distribution of immunotypes differed in the various serogroups. The serogroup A isolates (n = 13) were either L10 (1 isolate expressed both L10 and L11),

Table 6. Distribution of LOS immunotypes per serogroup among 562 meningococcal isolates recovered from patients with meningococcal disease in the Netherlands, 1989-1990 (percentages of immunotype in indicated category between brackets)

	Serogroup				
Immunotype	A	B	C	Other	Total
L1	0	3 (1)	0	0	3 (1)
L1,8	0	46 (11)	2 (2)	1 (5)	49 (9)
L2	0	42 (10)	31 (30)	7 (37)	80 (14)
L2,1	0	1 (0)	0	0	1 (0)
L3	7 (54)	151 (36)	39 (37)	7 (37)	204 (37)
L3,1	0	16 (4)	1 (1)	1 (5)	18 (3)
L3,1,8	0	7 (2)	3 (3)	0	10 (2)
L3,8	1 (8)	115 (28)	7 (7)	0	123 (22)
L4	0	16 (4)	16 (15)	2 (11)	34 (6)
L8	0	19 (5)	3 (3)	0	22 (4)
L8,10	0	2 (0)	0	0	2 (0)
L10*	4 (31)	0	0	0	4 (1)
Nontypeable	1 (8)	0	3 (3)	1 (5)	5 (1)
Non-interpretable		7			7
Total	13	425	105	19	562

* includes 1 L10,11 isolate

or L3 or related IT, and 1 isolate was NT. In serogroup B (n = 425), 7 isolates showed an non-interpretable IT result. L3 and the related immunotypes L3,1, L3,1,8 and L3,8 predominated (69%), and L2 and L4 accounted for 10% and 4%, respectively. In contrast, almost half of the 105 serogroup C isolates were L2 or L4 (30% and 15%, respectively). L1, L8 and L1,8 comprised 17% of the serogroup B and 5% of the serogroup C isolates. The other serogroups, which were only scarcely represented, harboured various ITs.

Association of immunotype and (sero)subtype

Of the 13 serogroup A isolates, 7 were A:NT:P1.16:L3. The two A:4:P1.10 isolates were L10 and L10,11, respectively, and of the 3 A:4:P1.9 isolates, 2 were L10 and 1 was NT. The exotic A:15:NT isolate was L3,8.

Within both serogroups B and C, L2 and L4 were strongly associated with the serotypes 2a and 2b. Of the 15 B:2a isolates, 3 were L2 (20%) and 10 were L4 (67%), and of the 44 C:2a isolates, 18 were L2 (41%) and 10 were L4 (23%). L2 and L4 were found in 45% and 9% of B:2b isolates (n = 11), and in 31% and 8% of C:2b isolates (n = 13), respectively. Among the serotype 2a and 2b isolates of those 2 serogroups, no L1, L1,8 or L8 was found. L3 and related ITs were present in 124 of 181 B:4 isolates (69%) and in 38 of the 43 B:15 isolates (88%), whereas the percentage of the L1/L8 category of ITs was 25% and 7%, respectively. Serogroup B isolates with subtype P1.4, P1.5, P1.15, P1.16 or P1.7,16 were mainly of the L3 and L1/L8 category of ITs, ranging from 71-91% and 0-26%, respectively, but those with the subtypes P1.2 or P1.5,2 were mainly associated with L2 (57% and 15%) and L4 (14% and 30%). The latter association also applies for serogroup C: all 3 P1.2 isolates were L2, and of the 36 P1.5,2 isolates, 17 were L2 (47%) and 8 were L4 (22%). Of the 16 subtype P1.5 isolates of serogroup C, L3 and related ITs were found in 69%. Among the 116 isolates of the most prevalent phenotype (B:4:P1.4) in the Netherlands, only 3 (3%) were L2, 83 (72%) were L3 or related IT, and 30 (26%) were of the L1/L8 category. For the 31 isolates of the "Norwegian" phenotype B:15:P1.(7,)16, these percentages were 6%, 94% and 0%, respectively.

The other serogroups were heterogenous with regard to both serosubtype and IT.

DISCUSSION

The various surface structures of *N. meningitidis* are useful tools for studying the spread of meningococcal disease. In the past decade a number of moabs against LOS have been developed. This has enabled immunotyping by WCE to be performed easily and on a large scale, but despite this improvement only a few studies concerning *N. meningitidis* actually include LOS ITs. A reason for this might be the absence of specific moabs for some ITs, such as L2 and L4. In our study, the moabs Mn15A8-1, Mn15A17F12, and 9-2-L3,7,9 were found to react with the L2 reference strain and moab Mn15A8-1 with the L4 reference strain. These moabs, in combination with 11

other moabs, enabled us to develop an algorithm for the assignment of LOS ITs of meningococci, by which the ITs are assigned on the basis of the reaction pattern of the isolates to these moabs. ITs L1, L5, L6 and L10 are easily recognizable, because specific moabs are available for these ITs. Moabs Mn4A8B2 and Mn14F20-11 were found suitable for determining L3 and L8, respectively. L3, L7 and L9, which are immunochemically identical,[15 27] could not be separated from each other by our moabs. Because the chemical structures of L7 and L9 are not yet known, isolates of this IT complex are labelled L3 for the time being. L2 and L4 are assigned by subtraction. Therefore, the assignment of L2, and especially L4, seems liable to error, because this is based on negative reactions with other moabs, apart from a positive reaction with moabs Mn15A8-1 and/or Mn15A17F12 and 9-2-L3,7,9. Inaccuracy in the laboratory can, therefore, easily lead to errors and misclassification. However, the immunotyping results obtained by WCE with monoclonal antibodies, using this algorithm, were similar to those obtained in an earlier study by microprecipitation with polyclonal antibodies.[15] A discordant IT result was obtained in only 3 out of 57 strains. Because of the lack of a gold standard, these differences might be due to errors in either of the 2 methods. However, for 2 of the strains the difference may be attributed to the microprecipitation method, because the ITs of 2 separate cultures of the same isolate, as determined by WCE, were identical. In 10 strains subtle differences occurred, pertaining to the presence of additional ITs among related ITs (see below). Unfortunately, no L4 strain was present among the isolates tested for comparability. The exact assignment of L4, therefore, remains uncertain.

The immunotyping results obtained by the use of the above-mentioned algorithm were reproducible. When comparing the ITs, as determined with a WCE of 2 separate cultures of the same isolate, only 5 discordant pairs among 116 pairs (4%) were found. In 3 of these pairs, one of the cultures resulted in L2 and the other in an non-interpretable result. The difference in the fourth pair (L4-L2) was inherent to the method used, and in the last pair a shift from L8 to NT was found, which is most likely due to an error in the laboratory. This also seems to have been the cause of 5 of the 7 minor differences between the paired test results. The remaining 2 minor differences, however, could well have been caused by plate effects which, therefore, seem to occur only rarely.

The algorithm described above is appropriate for LOS immunotyping of serogroup B and C meningococci. For serogroup A, however, the scheme is less suitable, as for L11 and the recently discovered ITs L12 and L13 no moab is yet available.[3] The assignment of L11 is possible, but rather complicated. There were, however, remarkable findings among the 13 serogroup A meningococci in our studies. Of the 5 group A isolates with a typical group A serotype-subtype combination (4:P1.9 and 4:P1.10), 4 isolates expressed the L10 IT and 1 was NT. The 8 remaining serogroup A isolates were of a serotype (serotype 15) or subtype (P1.16), which are typical for serogroup B, as are L3 and L3,8, which are also expressed by these isolates. This suggests a recent switch of the capsule of strains that were originally serogroup B to group A.

Based on the reaction patterns found among serogroup B and C meningococci, 2 main categories of related ITs can be found within these serogroups: one category is L2/L4, and the other L3/L1/L8. In view of the chemical structure of the different ITs (Figure 1), it is conceivable that an L3 strain is also able to express L1, L8, or both, because the terminal structures of these 3 ITs differ only slightly.[5 6 8 9 21] Considering the reaction patterns of isolates belonging to the L3/L1/L8 category, all L1 and many L3 strains reacted weakly with the L8 group of moabs, and many L8 strains reacted weakly with the L3 group. The differences in this category, therefore, seem to be of a quantitative nature, rather than a qualitative one. This implies the existence of a related group of L3, L1 and L8 ITs with subtle or more pronounced IT varieties and an understandable shift of ITs. In this respect, it is remarkable that in Great Britain a major difference has been discovered between carrier isolates (mostly L1/L8) and case isolates (L3).[10]

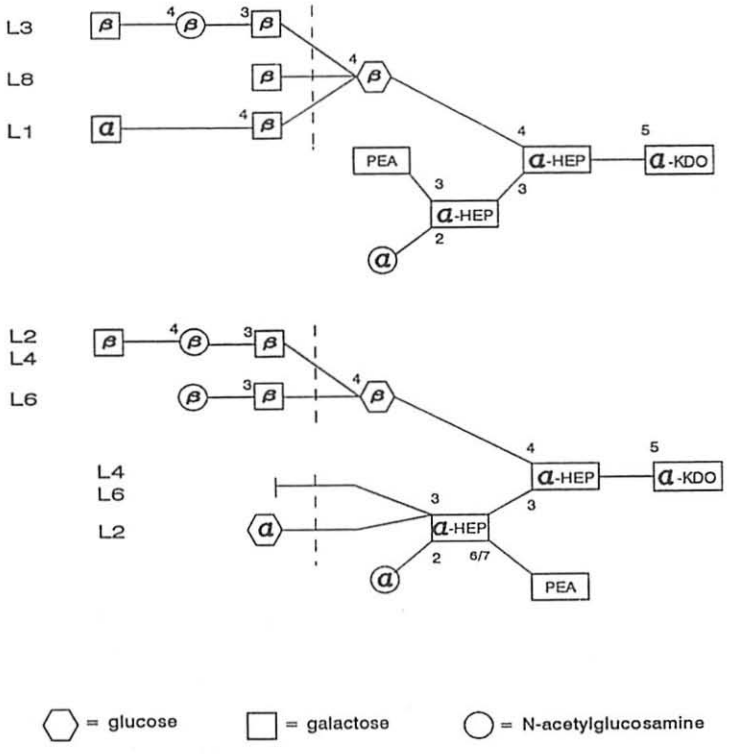

Figure 1. Primary structure of meningococcal oligosaccharides of immunotypes L1, L2, L3, L4, L6 and L8. Differences between the various immunotypes are presented on the left of the dotted line. The arabic numbers indicate the position of the binding site. α,ß indicate the anomeric configuration. KDO = 2-keto-3-deoxyoctulosonic acid; PEA = phosphoethanolamine; HEP = heptose. The structures presented are based on references 5, 6, 8, 9 and 21

The reference strains of the L3/L1/L8 group of ITs did not show "pure" reaction patterns: strain 126E (L1) also reacted with the L8 group of moabs, strain M978 (L8) with the L3 group and 6275 (L3), though weakly, also with the L8 group (Table 1). None of our isolates showed a "pure" L1 reaction pattern, but strain no. 892385 (Table 3) reacted solely with the L3 group of moabs. Strain no. 900714 (Table 3) seems to perform better than the original L8 reference strain M978 (Table 1), although it reacted weakly with 2 of the L3 group of moabs. The same reasoning may apply to strain no. 900100 as a reference for L10. Therefore, the above-mentioned strains from our series could, for the time being, be used as new references until new reference strains which express a single immunotype have been constructed by recombinant DNA technology.

With the chemical structure in mind, the dissimilarity between the L2/L4 and L3/L1/L8 categories is based on the location of the phosphoethanolamine group, and a shift of ITs between those 2 categories is very unlikely. We have not found ITs of these 2 unrelated categories in the same pair of any of the 116 isolates which were cultured twice. Among the 112 pairs of isolates, obtained from both the blood and CSF of the same patient, in the only instance in which the CSF isolate (L2) and the blood isolate (L1,8) of the same patient belonged to those unrelated categories, the reaction patterns of the 2 isolates differed completely, and in such a way that an erroneous interchange of isolates of 2 different patients seems to be the most likely explanation. The occurrence of L1 among L2 or L4 isolates is theoretically possible. We have found this combination only rarely, and we somewhat doubt its true existence.

From 1989 to 1990 L2, L3 and L4 were the most prevalent ITs among serogroup B and C meningococci in the Netherlands. Among these 2 serogroups, 2 main categories of related serotypes, subtypes and immunotypes could be distinguished during this period: the serotypes 4 and 15, the subtypes P1.4, P1.5, P1.15, P1.16 and P1.7,16, and the immunotypes of the L3/L1/L8 category on the one hand, and the serotypes 2a and 2b, the subtypes P1.2 and P1.5,2, and the immunotypes L2 and L4 on the other. The first category was typical for serogroup B, and the second for serogroup C. The B:4:P1.4 phenotype, which is at present the most prevalent in the Netherlands,[22] belonged almost exclusively to the L3 complex, which also applies to the "Norwegian" B:15:P1.7,16 phenotype in the Netherlands.[17]

The LOS IT is considered to be a virulence determinant.[10] Isolates of ITs with a terminal lacto-N-neotetraose unit (L2, L3, L4, L5, L7 and L9) are capable of endogenously sialylating their LOS, which renders them seroresistent by down-regulating the alternative complement pathway.[13 25] L1, L6 and L8 strains are not structured in this way, and are serosensitive.[13 25] Theoretically, strains of the latter ITs will be isolated more often among carriers, as was indeed the case in Great Britain.[10] However, a relatively large proportion (13%) of L1, L1,8 and L8 strains, not or only weakly expressing L3, was found among our patient isolates in the period 1989 to 1990. It might be possible that a carrier strain of the L1/L8 category shifts to the L3 group during colonization, which enables the isolate to evade the host defense

mechanisms and to cause disease. The high proportion of these L1/L8 isolates among our patient strains could be due to a similar shift from L3 to L1/L8. An association similar to the combination L3 and L1/L8 could be postulated for L2, L4 and L6, but we did not find any L6 isolates or a combination of L6 with L2 or L4. Unfortunately, no carrier strains were available for this study.

This study has demonstrated that it is possible to carry out meaningful LOS immunotyping of meningococci by the use of carefully selected moabs, despite the lack of specific moabs for L2, L4 and L11. The assignment of the latter ITs, therefore, is complicated and the development of specific moabs for these ITs is warranted. The method, however, is easily applicable for epidemiological research, and provides the possibility of better definition of test strains for *in vitro* bactericidal assays and pathogenesis studies. Future studies with respect to the elucidation of the genetic organization of LOS biosynthetic genes and LOS chemical structures are needed to further improve LOS epitope characterization. This will ultimately provide a potential for the construction of immunotype reference strains that express a single immunotype, the selection of appropriate moabs and the definition of sugar structures as targets for protective immunity.

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CHAPTER 5

PATIENT AND STRAIN CHARACTERISTICS IN RELATION TO THE OUTCOME OF MENINGOCOCCAL DISEASE

A multivariate analysis

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ABSTRACT

The aim of this prospective study, conducted in the Netherlands from 1989 to 1990, was to investigate the joint association of patient and strain characteristics with the outcome of meningococcal disease (MD). The study included 563 consecutive cases of systemic MD. Patient data were collected by means of a questionnaire. The meningococcal isolates were characterized with regard to their surface characteristics. Sequelae occurred in 8.5% of the patients, and were only associated with the presence of septicemia. The case-fatality rate was 7.7%. In a multivariate analysis, age, gender and the clinical presentation (meningitis and/or septicemia) were found to be the most important determinants for a fatal outcome. Infants aged ≤5 months and patients 10-19 years and ≥50 years of age had an increased risk for a fatal outcome compared with children from 6 months to 9 years old (Odds Ratios (OR): 5.1, 3.4 and 9.8, respectively). The OR for females versus males was 2.3. The risk for a fatal disease outcome was higher for patients with septicemia or a combination of septicemia and meningitis, compared with meningitic patients (ORs 2.3 and 3.1, respectively). Meningococcal strain characteristics did not influence the case-fatality rate substantially during the study-period. In conclusion, host factors were found to be determinants for a fatal outcome of MD in the Netherlands from 1989 to 1990.

INTRODUCTION

Meningococcal disease (MD) is a major health issue in both developing and industrialized countries. The reported case-fatality rates (CFRs) of MD range from 2% to 13%, and in 3% to 11% of the survivors serious sequelae are encountered.[1-9] Despite the improvement of medical care in the past 4 decades, little progress has been made in reducing the number of fatal cases of MD.[7]

Several surface characteristics of *Neisseria meningitidis*, such as the serogroup and serotype, have been found to be related with an unfavourable outcome of MD.[3 6 10] In the past decade an increasing number of monoclonal antibodies (moabs) for serotyping, subtyping and lipooligosaccharide (LOS) immunotyping have been developed, enabling us to characterize meningococci more accurately and to analyse in more detail the relation of strain characteristics with the disease outcome.[11 12] The outcome of MD, however, is also determined by host-related factors, such as the age of the patient and underlying diseases compromising the host defense mechanisms.[1-6 8 9] When determining the relation between a single determinant and the disease outcome, there is always the possibility that this relation is confounded by other determinants. By using multivariate statistical methods it is possible to adjust for the influence of other covariates and to assess the relation of individual variables with the disease outcome, conditional on the other variables.

In this paper we present the results of a multivariate analysis of the association of strain characteristics of *N. meningitidis* and patient characteristics, on the one hand, with the outcome of disease on the other, during a period of high endemicity in the Netherlands.[13]

PATIENTS AND METHODS

Patients

The study included 563 consecutive bacteriologically confirmed cases of systemic MD in the Netherlands, reported between 1st April 1989 and 30th April 1990. A case was defined as septicemic if *N. meningitidis* was cultured from the blood alone, and as meningitic if there was a positive culture from the cerebrospinal fluid (CSF) alone. Data on disease outcome (survived or deceased), the presence of sequelae, age, gender and predisposing factors were collected by means of a questionnaire, completed by the specialist in attendance. The presence of sequelae was assessed at least 4 weeks after admission to hospital. Sequelae were considered to be the occurrence of seizures, hydrocephalus, cerebral atrophy, mental retardation, hearing loss, paralysis of a cranial nerve, disturbance of vision, hemiparesis, peripheral neuropathy, scars after skin necrosis, amputation, behavioural disturbances and hypopituitarism. The following conditions were considered to be predisposing factors for a fatal disease outcome: severe head injury, a CSF leak, diabetes mellitus, chronic obstruc-

tive lung disease, chronic renal failure, liver cirrhosis, malignancy, immunosuppressive therapy and intravenous drug-abuse.

Bacterial isolates

Meningococcal isolates of all patients were submitted to the Netherlands Reference Laboratory for Bacterial Meningitis by regional laboratories. Pairs of isolates, cultured from both the CSF and blood of the same patient, were obtained from 123 cases. In 11 pairs, 1 isolate of each pair did not survive the transport to our laboratory. Both isolates of each of the remaining 112 pairs were identical with regard to the surface characteristics, and only 1 of each pair was included in the analysis.

Serogrouping and typing

Serogrouping was performed by means of Ouchterlony gel diffusion.[14] Serotyping, subtyping and LOS immunotyping were performed by means of a whole-cell ELISA, as previously described, with an extensive set of moabs.[11] The assignment of LOS immunotypes is described elsewhere.[12] It was not possible to assign an immunotype to 8 serogroup B isolates, due to an non-interpretable moab reaction pattern.

Statistical methods

The chi-square test was used for the analysis of contingency tables. Fisher's exact test was used when appropriate. The relation of the various variables with the outcome of the disease was analysed by means of a multiple logistic regression analysis. All variables which were associated with the disease outcome were included in the initial model. The model was fitted by backward elimination of individual variables. In the final step, interaction terms were added and tested for further improvement of the model. The assessment of the fit was performed as described by Hosmer and Lemeshow.[15]

In the statistical analyses the serogroups X, Y, Z, W-135 and 29E and nongroupable isolates were combined, because of their small numbers. For the same reason the serotypes 1, 14, 16 were combined, as were the subtypes P1.1, P1.6, P1.7, P1.7,1, P1.9, P1.10, P1.12, P1.14. The immunotypes were pooled in 4 categories: L1/8 (L1, L1,8, L8 and L8,10), L2/4 (L2 and L4), L3/1/8 (L3, L3,1, L3,1,8 and L3,8), and 'other' (L10, L10,11 and nontypeable isolates).

Statistical analyses were carried out on a personal computer using the SPSS/PC^R package.

RESULTS

During the study-period, 563 patients (295 males, 268 females) with systemic MD were reported. The outcome of disease was known for 562 patients, of whom 43 died (CFR = 7.7%).

Table 1. Distribution of cases and case-fatality rates (between brackets) of meningococcal disease in the Netherlands, 1989-1990, by serogroup and clinical presentation

Serogroup	Clinical presentation						Total	
	Meningitis		Septicemia		Meningitis and septicemia			
A	12	(16.7)	1	(.0)	0		13	(15.4)
B	255	(3.9)	74	(8.1)	96	(8.3)	425	(5.6)
C	50	(4.0)	30	(16.7)	25	(24.0)	105	(12.4)
Other	8	(12.5)	9	(22.2)	2	(50.0)	19	(21.1)
Total	325	(4.6)	114	(11.4)	123	(12.2)	562	(7.7)

The CFR according to serogroup and clinical presentation (meningitis, septicemia or both) is shown in Table 1. The lowest CFR was found among meningitic patients (χ^2 = 10.1; 2 degrees of freedom (df): P = .006). The CFR differed among the various serogroups (χ^2 = 11.7; 3 df: P = .009) and was highest in disease due to the uncommon serogroups (21.1%). These differences are at least partially due to an uneven

Table 2. Distribution of cases and case-fatality rates (between brackets) of meningococcal disease in the Netherlands, 1989-1990, by serotype and subtype according to serogroup

Variable	Serogroup								Total	
	A		B		C		Other			
Total no. of patients	13	(15.4)	425	(5.6)	105	(12.4)	19	(21.1)	562	(7.7)
Serotype										
2a	0	-	15	(6.7)	44	(11.4)	3	(.0)	62	(9.7)
2b	0	-	11	(9.1)	13	(15.4)	1	(.0)	25	(12.0)
4	5	(.0)	184	(4.3)	9	(22.2)	6	(16.7)	204	(5.4)
15	1	(100)	44	(4.5)	2	(.0)	0	-	47	(6.4)
Other	0	-	18	(5.6)	2	(50.0)	2	(.0)	22	(9.1)
Nontypeable	7	(14.3)	153	(7.2)	35	(8.6)	7	(42.9)	202	(8.9)
Subtype										
P1.2	0	-	7	(14.3)	3	(.0)	1	(.0)	11	(9.1)
P1.4	0	-	168	(3.6)	1	(.0)	2	(.0)	171	(3.5)
P1.5	0	-	9	(11.1)	16	(12.5)	4	(75.0)	29	(20.7)
P1.5,2	0	-	20	(5.0)	36	(13.9)	3	(.0)	59	(10.2)
P1.7,16	0	-	24	(8.3)	0	-	0	-	24	(8.3)
P1.15	0	-	24	(12.5)	2	(.0)	0	-	26	(11.5)
P1.16	7	(14.3)	43	(9.3)	0	-	1	(.0)	51	(9.8)
Other	5	(.0)	60	(3.3)	29	(10.3)	5	(20.0)	99	(6.1)
Nonsubtypeable	1	(100)	70	(5.7)	18	(16.7)	3	(.0)	92	(8.7)

distribution of the clinical presentation among the various serogroups (χ^2 = 22.8; 6 df: $P < .001$).

No significant association was found between the CFR and serotype or subtype (χ^2 = 3.1; 5 df: $P = .680$ and χ^2 = 13.1; 8 df: $P = .109$, respectively; Table 2). The serotypes 2a and 2b and the subtypes P1.2, P1.5, P1.5,2, P1.15 and P1.16 tended to have somewhat higher CFRs. Lower CFRs were found in patients with disease due to serotype 4 (5.4%) and subtype P1.4 (3.5%), which are at present the most prevalent serotype and subtype in the Netherlands.

The association between the CFR and LOS immunotype for each serogroup is shown in Table 3. Immunotypes L2 and L4 were associated with the highest CFR (11.3%), but this association was not statistically significant (χ^2 = 3.7; 3 df: $P = .296$). Within serogroup B, the CFR in disease due to L2 and L4 (8.5%) was almost twice as high as that in disease due to the L3 group of immunotypes (4.8%), but among serogroup C isolates these CFRs were almost equal (12.8% and 14.0%, respectively).

Table 3. Distribution of cases and case-fatality rates (between brackets) of meningococcal disease in the Netherlands, 1989-1990, by immunotype and serogroup

Immunotype category	Serogroup A		B		C		Other		Total	
L1/8	0	-	69	(5.8)	5	(.0)	1	(.0)	75	(5.3)
L2/4	0	-	59	(8.5)	47	(12.8)	9	(22.2)	115	(11.3)
L3/1/8	8	(25.0)	289	(4.8)	50	(14.0)	8	(25.0)	355	(7.0)
Other	5	(.0)	0	-	3	(.0)	1	(.0)	9	(.0)
Total	13	(15.4)	417	(5.5)	105	(12.4)	19	(21.1)	554	(7.6)

NOTE: In 8 cases due to serogroup B no immunotype could be assigned

Table 4 shows the age-specific CFRs, according to gender. The highest CFR is found among patients over 50 years of age (29.7%), followed by infants aged 0-5 months (16.7%) and teenagers (10.3%; χ^2 = 38.3; 4 df: $P < .001$). The CFR among female patients (11.2%) differed significantly from the CFR among males (4.4%; χ^2 = 9.3; 1 df: $P = .002$). Female patients were older on the average (χ^2 = 7.9; 4 df: $P = .094$). The largest gender-specific CFR differences were found in the age-categories 10-19 and ≥50 years of age, in which females outnumbered males (Table 4). There was no significant association between gender and serogroup (χ^2 = .1; 3 df: $P = .996$) or clinical presentation (χ^2 = .0; 2 df: $P = .993$).

There were 26 patients (4.8%) with a predisposing factor. The CFR among those patients (19.2%) was almost 3 times higher than that of patients without a predisposing factor (6.7%; Fisher's exact test: $P = .034$). Predisposed patients were found to suffer more often from septicemia than other patients (χ^2 = 11.2; 2 df: $P = .004$).

Table 4. Distribution of cases and case-fatality rates (between brackets) of meningococcal disease in the Netherlands, 1989-1990, by age-category and gender

	Gender					
Age-category	Male		Female		Total	
0-5 months	12	(16.7)	12	(16.7)	24	(16.7)
6 months - 9 years	166	(2.4)	123	(4.9)	289	(3.5)
10-19 years	73	(4.1)	83	(15.7)	156	(10.3)
20-49 years	30	(3.3)	26	(3.8)	56	(3.6)
≥50 years	14	(21.4)	23	(34.8)	37	(29.7)
Total	295	(4.4)	267	(11.2)	562	(7.7)

The proportion of patients with a predisposing factor was highest among patients over 50 years of age (37.8%; $\chi^2 = 100.0$; 4 df: $P < .001$). Predisposed patients and patients over 50 years of age suffered significantly more often from disease due to the uncommon serogroups ($\chi^2 = 21.8$; 3 df: $P < .001$ and $\chi^2 = 49.0$; 12 df: $P < .001$, respectively).

In the multivariate analysis, only the clinical presentation, age and gender turned out to be significantly associated with a fatal disease outcome, and predisposing factors and strain characteristics did not influence the CFR substantially. No significant interactions between the covariates were found. The estimated adjusted Odds Ratios (ORs) for a fatal outcome for each (category of a) variable with respect to a selected reference category in the final model are shown in Table 5.

Table 5. Adjusted Odds Ratios (OR) for fatal outcome for variable versus the reference category of each variable (OR = 1.0) for 562 patients with systemic meningococcal disease in the Netherlands, 1989-1990

Variable	OR	(95%-Confidence Interval)
Age of patient		
0-5 months	5.1	(1.4 - 18.2)
6 months - 9 years	1.0	
10-19 years	3.4	(1.5 - 7.9)
20-49 years	1.1	(.2 - 5.1)
≥50 years	9.8	(3.6 - 26.2)
Gender of patient		
Male	1.0	
Female	2.3	(1.2 - 4.7)
Clinical presentation		
Meningitis	1.0	
Septicemia	2.3	(1.0 - 5.3)
Meningitis and septicemia	3.1	(1.4 - 6.8)

The striking difference in the risk of fatal disease for female versus male patients still stands after adjustment for the other covariates (OR = 2.3; 95%-confidence interval (95%-CI): 1.2 - 4.7).

Serious sequelae were reported in 44 of the 515 surviving patients (8.5%). In 4 survivors the presence or absence of sequelae was not specified. Five patients had 2 sequelae and 4 patients more than 2 sequelae. Scars after skin necrosis were reported in 20 patients, and amputation in 4. Hearing loss (16 patients), paralysis of a cranial nerve (5), hydrocephalus (3), cerebral atrophy (2) and seizures (2) were the most frequent neurological sequelae. Other sequelae were mental retardation (3 patients), disturbance of vision, hemiparesis, peripheral neuropathy, hypopituitarism and behavioural disturbance (each in 1 patient). The distribution of sequelae among the various serogroups is shown in Table 6. The absence of patients with sequelae in disease due to the uncommon serogroups is noticeable. There was no association of the occurrence of sequelae (either combined or separated into neurological and hemodynamic sequelae) with serogroup, serotype, subtype or immunotype of the causative agent, or with the gender or age of the patient. Sequelae occurred in 6.5% of the meningitic patients, in 9.0% of the septicemic patients, and in 13.9% of patients with both meningitis and septicemia ($\chi^2 = 5.6$; 2 df: $P = .061$). The sequelae rate among patients with a predisposing factor (19.0%) was higher than among those without (8.3%; Fisher's exact test: $P = .100$). In a multiple logistic regression model, only the clinical presentation was significantly associated with the occurrence of sequelae. The OR for the occurrence of sequelae for septicemic versus meningitic patients was 1.4 (95%-CI: .6 - 3.2), and for patients with both septicemia and meningitis versus meningitic cases 2.3 (95%-CI: 1.1 - 4.7).

Table 6. Sequelae among 515 patients with systemic meningococcal disease in the Netherlands, 1989-1990 according to serogroup (percentage of patients with sequelae in indicated serogroup between brackets)

	No. of patients (percentage of sequelae)					
Sero-group	No. of survivors	Hearing loss	Damage of other cranial nerve/mot. disturbance	Other neurological or psychological disturbance	Scars and/or amputation	Total*
A	11	1 (9.1)	1 (9.1)	0 -	0 -	2 (18.2)
B	399	12 (3.0)	4 (1.0)	6 (1.5)	16 (4.0)	38 (9.5)
C	90	3 (3.3)	1 (1.1)	0 -	4 (4.4)	8 (8.9)
Other	15	0 -	0 -	0 -	0 -	0 -
Total	515	16 (3.1)	6 (1.2)	6 (1.2)	20 (3.9)	48

* some patients had more than 1 sequela

DISCUSSION

The overall CFR during the study-period was 7.7%, which is in agreement with the CFRs reported in the literature.[1-9] Our calculation was based on bacteriologically confirmed cases. Because diagnostic procedures concerning severely ill patients are prone to errors, it is likely that, especially in fatal cases, the diagnosis will not be bacteriologically confirmed, and the observed CFR of 7.7% may be an underestimation of the true CFR of MD in the Netherlands. Indeed, during the study-period another 52 patients were reported, of whom the diagnosis was based on clinical grounds (n = 37), or from whom the cultured isolate had not been submitted or did not survive during transport to our laboratory (n = 15). The CFR in this group was 18%, indicating a reporting bias of fatal cases. The CFR calculated on all known patients during the study-period would be 8.5%.

The CFR of 7.7%, which was determined in our prospective study, is higher than the CFR of 5.1%, which was found in an earlier, retrospective study in the Netherlands from 1959-83.[6] The calculation of the latter CFR was based on hospital records and may be underestimated, because in a retrospective study records of deceased patients may be missing. This might explain the difference.

In our series, age, gender and the clinical presentation were found to be the most important determinants for a fatal outcome of MD. Infants aged 0 to 5 months, teenagers from 10 to 19 years and adults over 50 years of age have an increased risk for a fatal disease outcome compared with children aged 6 months to 9 years, as has also been found in other studies.[3 5 6] The increased risk for infants and for adults over 50 years of age might be due to a failure of the immune system, the former due to immaturity and the latter due to normal decline.[16] The increased risk for teenagers might be related with a change in life-style during puberty, which could lead to a temporal deficit of the non-specific immunity.[16]

Male patients outnumbered female patients, which is usually found among cases of MD, but female patients were found to have an increased risk for a fatal disease outcome, even after controlling for possible confounding by age and the clinical presentation. We have no explanation for this finding. Of course, this association could be an instance of a false positive result, determined by chance alone. The excess fatalities among females, however, was also found among the additional 52 cases which were reported during the study-period (CFR for males 14.3% and for females 22.7%) and a similar difference was observed among 327 cases which were reported during the 7 months preceding the study-period, and from whom the disease outcome was assessed retrospectively (unpublished observations; CFR for males 8.4% and for females 10.4%). Therefore, this difference of CFRs between males and females seems to be valid for the Netherlands. However, this finding is not supported in the literature.[1 4] Only in the earlier-cited study concerning the period 1959-83 in the Netherlands, was the CFR for females slightly higher than for males, but this finding was not statistically significant.[6] In our series, the excess of fatalities among females is mainly determined by an excess number of fatal cases in the age-categories

10-19 and ≥50 years, in which females also outnumbered males. Women in these age-categories undergo major biological (hormonal) changes. It could be postulated that these changes might compromise the non-specific defense mechanisms among women which, in turn, could lead to an increased risk for contracting MD and a fatal outcome.[16]

The clinical presentation of MD (meningitis, septicemia, or both) turns out to be a third important determinant for a fatal disease outcome. The classification of patients into these 3 categories, which is generally found in reports concerning MD, is based on the source of isolation of the submitted isolate(s). Considerable bias, however, is to be expected, because in most cases it is not known whether specimens of both blood and CSF were investigated in the first place, and, in the case of a positive culture from both sources, whether indeed both isolates have been submitted. Therefore, a misclassification could easily occur. Notwithstanding this potential misclassification, major and consistent differences are found in the literature between the CFRs in these 3 categories, indicating that this 'surrogate' classification is a relatively good estimate of the true clinical presentation.[1-6 8 9] The concept underlying the clinical presentation is difficult to understand. It will be determined by both strain and patient characteristics, the latter seeming to be the most important factors. If an individual is suffering from an underlying disease which compromises his defense mechanisms, the invading pathogen can more easily cause septicemia, with or without meningitis. Predisposed patients were, indeed, found to suffer more often from septicemia than non-predisposed patients. In the multivariate analysis, however, the presence of a predisposing factor did not influence the risk for a fatal outcome of MD after adjustment for the clinical presentation and age. Other (non-specific) predisposing factors, which we did not or could not measure, could account for the strong association of the clinical presentation and age with the outcome.[16 17]

Of the various strain characteristics of *N. meningitidis*, only the serogroup was found to be related with a fatal outcome of MD in the bivariate analysis, but after adjustment for age, gender and the clinical presentation in the multivariate analysis, the influence of this strain characteristic on the disease outcome disappeared, indicating confounding by the other factors. This may also apply to the associations that were found in other studies.[3 6 10] It is not very likely that the meningococcal surface characteristics *per se* are determinants of a fatal disease outcome. However, it can not be excluded that in some circumstances a meningococcal clone exists with another underlying, but yet unknown factor that is associated with a fatal outcome (e.g. the amount of endotoxin-release).[18 19] If such a clone is homogenous with regard to the surface characteristics of *N. meningitidis*, an apparent association of one or more of these characteristics with the disease outcome will be found.

Sequelae were found in 8.5% of the survivors, which is similar to the findings of other reports.[3 6 7] In a multivariate analysis, the occurrence of sequelae was only related with the clinical presentation and not with strain characteristics, age, gender or predisposing factors.

In conclusion, host factors were found to be the most important determinants for a fatal outcome of MD in the Netherlands from 1989 to 1990. Apart from general measures, like the prompt initiation of antimicrobial treatment,[20 21] the only way to reduce the number of fatalities due to MD is to prevent the disease, i.e. by vaccination. Unfortunately, there is no vaccine currently available against meningococci of serogroup B, which is the most prevalent serogroup in industrialized countries.[2] The development of a serogroup B vaccine is mainly based on the class 1 outer membrane proteins, which determine the subtype of the meningococcus.[22] Restriction of the composition of the vaccine to the most prevalent meningococcal determinants might not be advisable, because host factors seem to be the most important determinants for the outcome of MD. Therefore, in order to protect highly susceptible patients as well, the future vaccine should also be directed against less prevalent meningococci, which can act as opportunistic pathogens.

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CHAPTER 6

SECONDARY CASES OF MENINGOCOCCAL DISEASE IN THE NETHERLANDS, 1989-1990

A reappraisal of chemoprophylaxis

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ABSTRACT

Objectives: To assess the secondary attack rate (SAR) of meningococcal disease (MD) among the household contacts of primary patients with MD and to describe the use of chemoprophylaxis in the Netherlands.
Design: Descriptive, nation-wide survey.
Patients and methods: Patients with MD, reported between 1st April 1989 and 30th April 1990, and their household contacts. A household contact suffering from MD between 24 hours and 1 month after hospital admission of the primary case, was considered to be a secondary case. Chemoprophylaxis was considered appropriate if rifampicin or minocycline had been prescribed to all household contacts within a maximum of one day after admission of the primary patient.
Results: The SAR was 0.3%. Chemoprophylaxis was prescribed to 627 of 1130 household contacts (55%). In 46% of those, the prophylaxis was considered appropriate. Of the 5 secondary cases, 2 were not given any prophylaxis, 2 received penicillin and 1 rifampicin. Of the primary patients, 6% was given prophylaxis during their stay in the hospital. All meningococci, isolated from pairs of primary and secondary patients, were rifampicin-sensitive.
Conclusions: The SAR of MD in the Netherlands is similar to that in other countries. Although the prescription of chemoprophylaxis is not recommended by the government, it is prescribed to 55% of the household contacts, and in almost half of these instances it was considered to be appropriate. Chemoprophylaxis is rarely prescribed to primary patients. Recommendations concerning chemoprophylaxis in the Netherlands, compared with standards elsewhere, are inappropriate and are in need of reappraisal. Based on results from this study and the literature, the prescription of chemoprophylaxis to the household contacts of a patient with MD, as well as to the primary patient, is recommended.

INTRODUCTION

The household contacts of a patient with meningococcal disease (meningitis and/or septicemia) have an increased risk of contracting the disease.[1-3] To protect these household contacts against secondary disease, chemoprophylaxis is recommended in the United States and Great Britain.[4,5] The objective of this measure is to eliminate meningococci from the nasopharynx of carriers, by which the further transmission of the meningococcus within the household is interrupted. It is assumed that this will prevent secondary cases of meningococcal disease (MD). The most preferred drug for chemoprophylaxis is rifampicin. Alternative drugs are minocycline, ceftriaxone and ciprofloxacin. These antibiotics are capable of eliminating meningococci from the nasopharynx (efficacy as compared to placebo: 79-100%).[6-8] Because most secondary cases occur within 5 days after hospital admission of the primary patient,[2] the usual recommendation is to prescribe the prophylaxis to the household contacts as soon as possible after diagnosis.[4,5] Because most antibiotics which are used for treatment of generalized MD do not reach sufficient salivary concentrations, it is recommended to give prophylaxis to the primary patient as well, prior to the patient's discharge from hospital, to ensure that carrier strains are not reintroduced into the household by the primary patient.[4,5,9] Medical personnel that has been in close contact with a patient with MD (mouth-to-mouth resuscitation), should also be prophylactically treated.[4,5]

In the Netherlands the prescription of chemoprophylaxis is not accepted practice. In 1980 the National Health Council preferred close monitoring of the health-status of contacts of a primary patient, and left the prescription of chemoprophylaxis to the opinion of the attending physician.[10] Apart from the advocation of rifampicin, no further instructions were provided with regard to chemoprophylaxis. In 1988 the prescription of chemoprophylaxis with rifampicin to the household contacts of a primary patient with MD was recommended in the Dutch journal "Nederlands Tijdschrift voor Geneeskunde", but in a subsequent paper in this journal no unified policy could be established with regard to chemoprophylaxis.[11,12] In 1990 it was concluded, again in this journal, that there was too little evidence of the beneficial effect of chemoprophylaxis with rifampicin to justify its prescription.[13] In the protocol "Infectieziekten" issued by the Department of the Chief Medical Officer of Health (CMOH), to which the Dutch Public Health Services refer, chemoprophylaxis with rifampicin is suggested, but not officially recommended.[14]

During the period from 1st April 1989 to 30th April 1990, a nation-wide survey on MD was conducted in the Netherlands. Secondary study-objectives were the assessment of the secondary attack rate (SAR) of MD among the household contacts of primary patients with MD, and the inventarization of the application of chemoprophylaxis in the Netherlands. In this article we present the results of this part of the survey, and advocate a reappraisal of the recommendation of chemoprophylaxis in the Netherlands.

PATIENTS AND METHODS

This study includes all patients with MD, from whom the causative meningococcus or other diagnostic material had been forwarded to the Netherlands Reference Laboratory for Bacterial Meningitis (RLBM) in Amsterdam between 1st April 1989 and 30th April 1990, or who had been reported otherwise during that period. For logistic reasons the study was interrupted during the summer months from July to September 1989. A patient was considered to be suffering from MD if *Neisseria meningitidis* was isolated from the cerebrospinal fluid (CSF) and/or blood (n = 471), meningococcal capsular polysaccharide-antigens were present in the CSF (n = 9) or there was a clinical syndrome of meningitis and/or septicemia with fever and a petechial rash, considered as MD by the attending physician (n = 22). After having obtained informed consent of the patient, or a parent or guardian of the patient, clinical data and data regarding recent contact with another patient with MD and the use of antimicrobial drugs prior to admission to hospital were provided by the specialist in attendance.

Persons who slept in the same house as the primary patient during the week prior to hospital admission of the patient were considered as household contacts. Every household was visited by a regional Public Health Officer as soon as possible after hospital admission of the primary patient, and each household member was asked to complete a questionnaire which contained questions on the composition of the household and the use of chemoprophylaxis. During another home-visit, approximately one month later, the household contacts were asked if they had contracted diseases, among which MD, since hospital admission of the primary patient.

A secondary case of MD was defined as the occurrence of MD in a household contact if it was diagnosed during the period from 24 hours after hospital admission of the primary patient to the time of the second home-visit to the household. Those household contacts who contracted MD within 24 hours after the primary patient was diagnosed, were defined as co-primary.

Chemoprophylaxis was considered optimal if rifampicin or minocycline had been prescribed to all household contacts within 24 hours after hospital admission of the primary patient.

All meningococci isolated from the primary and their secondary patients were tested for rifampicin-sensitivity by the disk-diffusion method.

RESULTS

Incidence and secondary attack rate

During the study period of 10 months, 502 primary patients with MD were reported. Among those were 2 pairs with co-primary patients, who were admitted to hospital within a few hours after each other (Table 1). During this period the incidence rate of MD was 4.0 per 100,000 person-years. There were 5 secondary cases of MD. The

Table 1. Co-primary and secondary cases of meningococcal disease among the household contacts of primary patients in the Netherlands, 1989-1990

Primary patient		Co-primary or secondary patient			
Household no.	Meningococcal phenotype*	Time-interval (in days)	Relationship	Meningococcal phenotype*	Prophylaxis
		Co-primary:			
-	B:4:P1.4	< 1	brother	B:4:P1.4	
-	B:NT:P1.16	< 1	sister	-†	
		Secondary:			
1	-†	2	sister	B:16:P1.12	none
2	B:4:P1.4	4	parent	B:4:P1.4	penicillin
3	B:4:P1.4	4	brother	B:4:P1.4	none
4	B:NT:P1.4	13	brother	B:NT:P1.4	amoxycillin
5	C:2a:P1.2	35	brother	C:2a:P1.2	rifampicin
6	B:2b:P1.2	55	cousin	B:2b:P1.2	not applicable‡

* NT = nontypeable
† clinical diagnosis
‡ not applicable: secondary patient entered the household after the prescription of rifampicin

time-interval between the diagnosis of primary and secondary patients ranged from 2 to 35 days (Table 1). The average number of household contacts was 3, which gives an estimated SAR of 3 / 1506 = 0.3%. If there was a positive culture of both isolates of a pair of patients, the phenotypes of those isolates were identical (Table 1). These isolates were all sensitive to rifampicin. There was one family in which a secondary case was reported 55 days after admission of the primary patient (case 6). This case, which was not included in the calculation of the SAR, concerned a child from a family which had been living temporarily with the family of the primary patient. The child arrived from its home country one week after the primary patient was discharged from hospital. All other members of both families had been treated with rifampicin immediately after admission of the primary patient.

Application of chemoprophylaxis

Data were obtained from 1130 household contacts of 378 primary patients. Chemoprophylaxis had been prescribed to 627 persons (55%) in 220 families. Among those were 3 secondary patients who had received penicillin, amoxycillin and rifampicin, respectively. Of the 598 contacts who had mentioned the name of the antibiotic, 520 were given rifampicin and 1 minocycline (87%). Ceftriaxone and ciprofloxacin were not prescribed. Chemoprophylaxis was given within one day after hospital admission of the primary patient in 408 of the 617 cases (66%). The starting-date was not mentioned by 10 contacts. In 186 households all members received prophylaxis. The prophylaxis was optimal in 276 out of 599 instances (46%), among which 1 secondary

case. In 28 household contacts it could not been assessed whether the prophylaxis had been optimal. Of the 499 primary patients, of whom data were provided, 13 had received rifampicin during their stay in hospital, and in 16 patients ceftriaxone was included in treatment of the generalized disease, which means that 29 patients (6%) had been treated with an antibiotic which is capable of eliminating meningococci from the nasopharynx. Chemoprophylaxis had been prescribed in 2 families of these 29 patients, and no secondary cases were reported in these 2 families.

DISCUSSION

Since 1958, meningococci isolated from patients with MD in the Netherlands have been submitted to the RLBM in Amsterdam for further classification. The coverage rate of the submissions has been estimated to be approximately 80%,[15] but this rate might have been even higher during the study period, because of the intense involvement of the many clinical microbiologists, clinicians and Public Health Officers. However, it can not be excluded that primary, and also secondary cases of MD have been overlooked, especially when the diagnosis was based on clinical grounds alone.

During the study-period the SAR was 0.3%, which is similar to that in other countries.[16]

In the Netherlands, chemoprophylaxis is not recommended in the first instance, and its prescription is left to the opinion of the attending physician.[10] In our study we found that chemoprophylaxis had been prescribed to 55% of the household contacts of primary patients. In 46% of those who had received prophylaxis, the regimen was optimal (i.e. rifampicin, prescribed to all household contacts within 1 day after admission to hospital of the primary patient). According to recommendations prevailing in other countries, which include the prescription of chemoprophylaxis to the primary patient as well, almost all prophylaxis in the Netherlands should be considered as sub-optimal, because the primary patient had been included in only 2 of the families treated. The inclusion of the primary patient, however, is not mentioned in the recommendations of the Health Council or the CMOH.[10 14]

In 1 of the 5 households with a secondary case, the prescription of chemoprophylaxis was in accordance with Dutch standards. The primary patients were not treated. It might be possible that this enabled the meningococcus of the primary patient to re-enter the family. The long time-interval (35 days) between the primary and secondary case in the family treated with rifampicin suggests that rifampicin might have postponed the occurrence of the secondary case. In the more recent literature this shift to a later occurrence of secondary cases after the use of rifampicin is described.[3] In our opinion, the occurrence of secondary MD in household no. 6 should be considered exceptional, but it might be postulated for this household as well that the meningococcus has been reintroduced into the family by the primary patient.

With the CFR of 5-10% and the sequelae rate of approximately 10% in mind,[16] it seems worthwhile trying to prevent secondary cases of MD in the Netherlands as well. However, the beneficial effect of chemoprophylaxis in the prevention of secondary cases of MD has never been demonstrated by experimental research. Its recommendation in the United States and Great Britain is, in fact, based on observational research, similar to the research presented in this paper, which might have been biased by extraneous factors which could have been overlooked. Opponents of chemoprophylaxis rightly point out this shortcoming. In addition, chemoprophylaxis with rifampicin has drawbacks, such as the occurrence of side-effects and the development of resistance to rifampicin.[18] The side-effects of a 2-day course of rifampicin, however, are minor and the development of rifampicin-resistance does not affect the treatment of systemic MD, because rifampicin is never used for that purpose. Failures of chemoprophylaxis have been reported, and have mainly been attributed to insufficient attention being paid to recommendations, or to a possible reintroduction of the meningococcus into the family.[3] Failures of chemoprophylaxis might also be due to rifampicin-resistance. All meningococcal isolates forwarded to the RLBM in 1988 (n = 384) were rifampicin-sensitive (unpublished observations), as were the isolates obtained from the pairs of primary and secondary patients during the study-period.

According to advocators of chemoprophylaxis, sufficient evidence is present in the literature to justify its prescription. It seems plausible that the further transmission of meningococci within a household is interrupted due to the elimination of the carrier strains, thereby preventing secondary disease. In meningitis caused by *Haemophilus influenzae* the beneficial effect of rifampicin in the prevention of secondary cases has, indeed, been demonstrated.[19]

Regarding the SAR of MD in the Netherlands, which is similar to that in other countries, and the inadequacy of recommendations according to standards elsewhere, we advocate reappraisal of the recommendations for chemoprophylaxis in the Netherlands. If it is concluded that the beneficial effect of chemoprophylaxis has not been conclusively demonstrated, then steps should be taken to initiate a well-organized trial concerning the effects of chemoprophylaxis. Based on our experience from the survey presented in this paper, this would definitely be feasible in the Netherlands. However, should it be concluded that the prescription of chemoprophylaxis should still be left to the opinion of individual physicians, then the instructions should be amended: if the attending specialist considers it necessary to prescribe chemoprophylaxis, rifampicin must be prescribed to <u>all</u> household contacts as soon as possible after diagnosing MD in the primary patient (dose: 10 mg/kg twice daily for 2 days, with a maximum of 600 mg per dose; infants aged < 1 month: 5 mg/kg twice daily for 2 days). Prior to discharge from hospital, the primary patient should also be given prophylaxis. Under all circumstances, also after the prescription of chemoprophylaxis, one should be alert for signs of secondary disease. Patients receiving rifampicin should be warned about an orange discolourization of urine and tears (also non-reversible discolourization of soft contact-lenses) and the interference of

rifampicin with oral contraceptives. During pregnancy or when other contra-indications are present, one intramuscular injection of 250 mg ceftriaxone can be given as an alternative.

In our opinion, sufficient similarity exists between meningitis caused by *N. meningitidis* and *H. influenzae* to justify the extrapolation of the results of the above-mentioned study of Band *et al.* to MD.[19] Therefore, for the prevention of secondary MD, we advocate the prescription of chemoprophylaxis to the household contacts of a patient with MD, and also to the primary patient prior to discharge from hospital.

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CHAPTER 7

GENERAL DISCUSSION AND RECOMMENDATIONS FOR FURTHER RESEARCH

In this thesis several aspects of the epidemiology of meningococcal disease in the Netherlands have been addressed. The core theme was studying the spread of meningococcal disease in the Netherlands in the past decade in an attempt to explain the increased incidence in the 1980s. This was done by observing several characteristics of the pathogen, which took a central position in this thesis, and the host. The ultimate goal was to provide information which may assist the (future) prevention of meningococcal disease. In the following three sections the relevance of the results of the various chapters will be discussed in retrospect, and recommendations for further research will be given. The first section discusses the factors that are related to the increased incidence of meningococcal disease, the second section deals with aspects pertaining to the future vaccine, and the third with chemoprophylaxis.

Increased incidence of meningococcal disease in the Netherlands from 1980 to 1990

Epidemic or endemic?

Neisseria meningitidis is capable of causing epidemics of meningococcal disease (MD), and epidemics have been reported world-wide, as well as in the Netherlands.[1,2] During the 1980s the incidence of MD in the Netherlands increased gradually from 1.1/100,000/year in 1982 to 3.5/100,000/year in 1990. At the end of 1988, alarming reports appeared in the lay press, which gave rise to much concern among the public, and the question was, whether we had to fear another epidemic of MD.

It is difficult to give an exact definition of an epidemic. In a handbook on the epidemiology of bacterial infections the following definition is given:[3] "An epidemic is said to exist when an unusual number of cases of a disease occur in a given time period and geographic area as compared with the previous experience with that disease in that area". It is also stated that this definition is arbitrary and will vary from disease to disease. The problem is how to determine what should be considered as an unusual number. For the designation "epidemic of MD in the Netherlands" an arbitrary cut-off value of 4.0 cases per 100,000 inhabitants per year has been applied.[4] According to this definition, the increased incidence of MD in the Netherlands in the late 1980s can not be considered an epidemic, because it did not exceed this cut-off value. However, if the estimated 80% coverage rate of the submissions of meningococci to the Netherlands Reference Laboratory for Bacterial Meningitis (RLBM) is taken into consideration,[5] the true incidence in 1990 would have been around 4.3/100,000/year, which would then meet this criterion.

It can be argued that this nominal criterion for the definition of an epidemic of MD alone will not suffice, and that other aspects should be considered as well. One of these aspects is the rate of the increase in frequency of the disease, which depends on the duration of the incubation period of the disease. Most epidemics of MD, as reported in the past, showed a sudden steep rise in the number of cases in a short period of time, which is in agreement with the short incubation period of MD,[3] followed by a rapid decrease.[1,2,6] The 1966 epidemic in the Netherlands is a good

example of the above-mentioned course.[2 6] The gradual rise of MD in the 1980s in the Netherlands contrasts sharply with the described steep rise of MD epidemics, and renders the nomination "epidemic" for the current situation in the Netherlands unlikely.

In our opinion, another factor necessary for an exact definition of an epidemic of MD pertains to the causative organism. *N. meningitidis* shows a marked heterogeneity with regard to both the phenotype, which is determined by its surface structures, and the genotype.[7 8] During an epidemic, one would expect to find an epidemic strain of one single phenotype (or at the most a very limited number of different phenotypes) which belongs to a single genotype or a limited set of closely related genotypes. Apart from B:4:P1.4, which was the most prevalent phenotype in the Netherlands in 1990 (21% of all cases) and belonged to a complex of closely related clones (lineage III),[9] a variety of other phenotypes and clones has been found, which gives little further support for the label "epidemic" in the Netherlands in the late 1980s.

In conclusion, the situation concerning MD in the Netherlands in the late 1980s, with the gradual rise of the number of infections and a variety of meningococcal phenotypes and genotypes, is not in accordance with the definitions of an epidemic, and can best be described as hyperendemic.

Possible explanations for the increased incidence

Explanations for an increased incidence of an infectious disease in a particular geographical area can be sought in (a combination of) the pathogen, the host, or the environment.

With respect to the pathogen, a possible explanation for a rise of the incidence could be the emergence of a virulent strain of *N. meningitidis* in a susceptible population. Such a strain could be imported from outside the country or result from a mutation of a pre-existing strain. In both circumstances it is to be expected that the genotype and phenotype of that particular strain are homogenous, at least immediately after its appearance. Indeed, this has often been reported with regard to MD, including the 1966 epidemic in the Netherlands (strain B:2b:P1.2).[9 10] As was pointed out by Caugant *et al.*, a new meningococcal clone complex (lineage III) appeared in the Netherlands around 1980.[9] Initially, this complex consisted of one single phenotype (B:4:P1.4), but later an increasing number of other phenotypes was found in this complex, mainly characterized by the subtype P1.4 (Chapter 3). If it is assumed that all P1.4 isolates collected in the late 1980s belong to this new clone complex (Chapter 3), approximately 30% of all cases of MD in 1990 in the Netherlands are due to the emergence of a new clone complex. The remaining part of the increase is due to a variety of other clones and/or phenotypes, suggesting that additional factors are responsible for the increase.

The susceptibility of the host is a crucial factor in contracting an infectious disease. It has been postulated that the waxing and waning of the specific immunity of the host to the various antigens of the meningococcus in the general population is reflected in the frequently found cyclical occurrence of MD.[1 6 11] The emergence in a

particular population of a meningococcal strain with new surface characteristics will elicit specific antibodies and result in an increased herd immunity. This, in turn, will ultimately lead to a decreased circulation of the new meningococcal strain, which will then cause only a limited number of sporadic cases. After a certain period of time, however, this "slumbering" strain will again be able to cause an increasing number of cases, due to the addition of new generations of susceptibles to the population. This might be an explanation for the recent rise in frequency of meningococcal strains that were already present in the Netherlands.

In Chapter 2 the possible role of an increased migration of the population has been brought forward as an explanation for the increased number of cases of MD in the Netherlands, and this could be regarded as an environmental factor. The increased migration could facilitate the circulation, transmission, and acquisition of the locally prevailing meningococcal strains, and provide an additional explanation for the increased incidence and the variety of meningococcal phenotypes. The increased circulation of meningococci should then be reflected in the occurrence of a broad spectrum of specific antibodies against the various meningococcal surface antigens in the general population.

The increased incidence of MD in the late 1980s in the Netherlands, therefore, seems to be due to a combination of strain, host and environmental factors. Research on the role of the decreased herd immunity or the increased circulation in causing an increased number of cases of MD can be included in the serological part of this survey. In order to gather information on the protective immunity, serum samples have been collected from members of different subpopulations in the Netherlands, among which samples from army recruits. The latter represent a sample of adolescent males of the general Dutch population in 1990. In addition, we have at our disposal serum samples of age-matched males, collected in 1975 during a population survey in Zoetermeer, the Netherlands. The army recruits were born around 1970, and are expected to be exposed to the meningococcal phenotypes which were prevalent from 1970 to 1990, whereas the members of the Zoetermeer group, born around 1955, represent a cohort (possibly) exposed to the phenotypes that were prevalent from 1955 to 1975. Guided by the knowledge of the distribution of the various meningococcal surface antigens in the past 3 decades (Chapter 2), suitable antigens can be selected, and the two groups of people can be compared with regard to the occurrence of specific antibodies against the various meningococcal antigens. This will enable us to investigate the possible role of both the herd immunity and the apparent increased circulation in causing an increased incidence of MD.

Further support for the increased circulation theory can be given by prospectively investigating carrier rates in the open population, preferably in combination with serology. Evidence for an increased migration of the population as a cause for the increase can also be assessed by a case-referent approach, in which the migration of cases of MD should be compared with that of age-matched referents from families that are similar with regard to composition, age-distribution, social class, and place of residence.

Possible changes in the virulence of N. meningitidis

From 1980 to 1990 the proportion of isolates cultured from the blood alone (blood-strains) increased from 8% to 21%, which may indicate an increased virulence of the causative meningococci (Chapter 2). A similar, but more pronounced increase has been found in the percentage of notifications of meningococcal septicemia in the Netherlands, which increased from 19% in 1980 to 47% in 1990 (figures from the Department of the Chief Medical Officer of Health). Another indication for an increased meningococcal virulence could be the case-fatality rate (CFR) of 7.7% during the period 1989-1990 (Chapter 5), which was higher than the CFR of 5.1% in the period 1959-1981.[12] This difference might be explained by the increase in the proportion of blood-strains during the 1980s, but after adjustment for this covariate a difference still is present. A more likely explanation for this difference is probably an ascertainment bias. We have assessed the outcome of disease in a prospective survey, whereas the survey of the period 1959-1981 was retrospective, and could have failed to detect all deceased patients. This may have led to an underestimation of the CFR during the period 1959-1981.[12]

In Chapter 5 it was pointed out that, after adjustment for confounding by host factors, the outcome of MD was not associated with specific meningococcal surface characteristics. In addition, the increase of the proportion of blood-strains could not be attributed to the occurrence of a specific (new) meningococcal phenotype. Thus, no relation could be found between the apparent increase in virulence and the meningococcal surface antigens. It might well be possible that this increase is due to extraneous factors which are not related to the meningococcus, e.g. more blood-cultures were carried out in the late 1980s than in the early 1980s. However, the existence of another virulence factor, which is shared by meningococci of the various phenotypes, can not be excluded. This factor might be the amount of endotoxin-release from the meningococcus,[13] which could be investigated by comparing isolates of fatal and non-fatal cases, or those of septicemic and meningitic cases.

Shift in the age-distribution of patients with meningococcal disease

From 1980 to 1990 a shift in the age-distribution of patients with MD from younger to older age-categories was noted (Chapter 2). Similar shifts have been found in the past in the Netherlands and also in other countries, and have been attributed to coinciding changes in the distribution of meningococcal phenotypes.[4 14 15] The association has been explained as follows: immunity to the meningococcus is acquired by (repeated) colonization and/or carriage of both pathogenic and non-pathogenic *Neisseria* spp.[16] Children <5 years of age are not immune and have to build up specific immunity against the meningococcus. For these children the antigens of all prevalent meningococci are new. Both existing and newly-appearing meningococcal strains will cause MD among children of this age-category, and the distribution of meningococcal phenotypes among these cases will be similar to the distribution of phenotypes in the general population. The ≥5 year-olds, however, can be expected to have built up specific immunity against the antigens of the various

meningococci which were already circulating in the population, but they will not be immune to antigens of newly-emerging meningococci. The appearance of a new strain will thus lead to an increase in the number of cases in the older age-categories, whereas the number of cases due to existing strains will remain more or less constant. These older patients will then be relatively over-represented, and a shift in the age-distribution of patients with MD will be found. The shift in the age-distribution that occurred in the past decade could at least partially be attributed to the emergence of new meningococcal phenotypes, especially among serogroup B isolates. The extensive collection of meningococcal isolates of the RLBM, and the improved methods for characterizing meningococci, enable further testing of the hypothesis, as cited in this paragraph, e.g. by studying the age-specific incidence of MD of patients of different birth cohorts in relation to the occurrence of new meningococcal phenotypes.

Implications for a future serogroup B meningococcal vaccine

Secular changes in the surface characteristics of serogroup B meningococci

As in most industrialized countries, serogroup B has been the predominant serogroup in the Netherlands during the past 32 years (Chapter 2).[1 6] Many different serotypes and subtypes have been found among isolates of this serogroup, and from 1958 to 1990 substantial changes have occurred in their distribution (Chapter 2). In addition, phenotypic changes in particular meningococcal clones have been found to occur fairly rapidly (Chapter 3). This may have important consequences for the future prevention of MD by vaccination. A capsular polysaccharide vaccine against disease due to meningococci of the serogroups A and C is already available.[17 18] However, it needs to be improved in order to protect infants as well.[17 18] The B polysaccharide has proved to be poorly immunogenic, and other vaccine candidates were sought. The development of a serogroup B vaccine is mainly based on epitopes of the class 1 outer membrane protein (OMP) and the class 2/3 OMP of the meningococcus which determine the subtype and serotype, respectively.[17 18] Such OMP vaccines lead to subtype and serotype-specific immunity.[17-19] However, it has been demonstrated that the bactericidal capacity of the antibodies directed to epitopes of the class 2/3 OMPs depends on the growth conditions of the meningococcus, whereas antibodies elicited by epitopes of the class 1 OMP are bactericidal irrespective of the growth conditions.[19] Therefore, the development of a serogroup B vaccine is now mainly directed to the class 1 OMPs, and it will be of major importance to monitor the distribution of subtypes among serogroup B meningococci over time. Experimental serogroup B vaccines have been tested in Cuba, Chile and Norway.[20-22] These vaccines were based on a single strain (B:4:P1.15, B:15:P1.3 and B:15:P1.7,16, respectively). The results of the Cuban trial among children aged 11-16 years were very promising, but it should be kept in mind that the circumstances in Cuba, an isolated island population with a very homogenous meningococcal subtype distribution, were very favourable. They were confirmed in a case-control study of the efficacy of this vaccine in Brazil.[23] The

Brazilian study, however, showed poor reactions in younger children. The Chilean vaccine provided low-level protection only.[20] The efficacy of the vaccine in the Norwegian trial among teenagers was 57%, and the authors concluded that the effect was insufficient to justify a public vaccination programme.[22] In regard of the variety of subtypes among serogroup B isolates in the Netherlands, a multivalent OMP vaccine will be needed in this country. The changes in the distribution of subtypes among serogroup B meningococci, in concert with the rapidly occurring phenotypic changes of a clone, require adaptation of such an OMP vaccine from time to time, and a periodical revaccination of (some of) the population with the adapted vaccine will probably be necessary. In this respect, the occurrence of an epidemic due to a meningococcal clone with a new virulent subtype has to be feared. Because the increase in frequency of the new subtype P1.4 in the Netherlands during the 1980s was gradual, it would have been possible to adapt the vaccine. Further research on the development of multivalent OMP vaccines is needed, and because of the apparent age-dependent efficacy of the previously tested vaccines, phase III trials of these vaccines should be conducted among infants as well.

During the study period, the annual proportion of nonsubtypeable serogroup B meningococci varied from 12% to 26% (Chapter 2). In order not to be surprised by the recrudescence of a meningococcal strain with a yet unknown subtype, further development of new monoclonal class 1 specific antibodies is needed in order to be able to recognize as many subtypes as possible, so that they can eventually be included in the vaccine.

The meningococcal class 1 OMP harbours 2 variable regions (VR1 and VR2) which determine 2 mutually exclusive sets of subtypes.[24] The epitopes that determine the subtypes P1.5, P1.7, and P1.12 are located in VR1, and the epitopes of the remaining subtypes in VR2. Each meningococcus that has a class 1 OMP should therefore express a subtype combination. The subtype combinations P1.7,1, P1.7,16, P1.5,2 and P1.12,16 are well known. However, the majority of the meningococci analysed expressed a single subtype (Chapter 2) and should, therefore, be considered partly nonsubtypeable. Part of this nonsubtypeability might be explained by so-called "hidden epitopes". The existence of a hidden P1.7 epitope has been demonstrated for isolates of the subtypes P1.16 [25] and P1.4 (Poolman *et al.* unpublished observations). Further research on the existence of other hidden epitopes, and the role of these epitopes in eliciting protective antibodies, is needed.

Another matter of concern are meningococci that do not express a class 1 OMP *in vitro*. It has been estimated that approximately 3% of all serogroup B isolates submitted to the RLBM in 1990 did not express a class 1 OMP, and the suggestion was put forward that this phenomenon could also occur *in vivo*, which might interfere with the class 1 specific immunity.[26] The potential absence of a class 1 OMP, the variety of subtypes among serogroup B meningococci and the experience of the previous OMP vaccination trials, require further research on other serogroup B vaccine candidates. One of these is the lipooligosaccharide (LOS) moiety of the outer membrane of meningococci.[17 18 27] A limited number of different immunotypes was found among sero-

group B meningococci (Chapter 4), which confirms the results of previous reports.[28 29] A vaccine based on LOS immunotypes could theoretically be restricted to L2, L3 and L4, because only these immunotypes seem capable of causing disease.[30] Because the meningococcal LOS, or endotoxin, plays a key role in the induction of septic shock,[31-33] an additional advantage of a vaccine based on the LOS moiety is that this vaccine will probably elicit neutralizing anti-endotoxin antibodies, which could reduce the number of fatalities of MD.[18 27]

The relevance of lipooligosaccharide immunotyping for the characterization of N. meningitidis

In the past, LOS immunotyping has been hampered by the complexity of the methods involved.[28 29] Despite the lack of specific monoclonal antibodies (moabs) against the immunotypes L2 and L4, we were able to develop an algorithm for easy determination of the LOS immunotype of *N. meningitidis* of the serogroups B and C in a whole-cell ELISA, using a panel of 14 moabs (Chapter 4). The determination of the immunotypes L2 and L4, however, is complicated and subject to error, and the development of L2 and L4 specific moabs is needed. For the determination of immunotypes among serogroup A meningococci the algorithm is less suitable, because no specific moabs against most of the regular immunotypes of this serogroup exist.

For epidemiological purposes LOS immunotyping is probably of limited value. Isolates of serogroup A are homogenous with regard to both the immunotype and serosubtype, and studying the distribution of immunotypes will have little additional discriminating power.[29 34-36] This may also apply to the serogroups B and C, which harbour only a limited number of different immunotypes.[28 29 37] For research on the protective immunity to the meningococcus, however, the LOS immunotype is of major importance. The LOS immunotype is considered to be a virulence determinant and plays an important role in eliciting specific immunity to the meningococcus.[18 37] By using the immunotyping method, as described in Chapter 4, it is possible to give a better definition of test strains for *in vitro* bactericidal assays and pathogenesis research, but further studies are needed in order to improve on LOS epitope characterization.

Chemoprophylaxis in the prevention of secondary cases of meningococcal disease

The continuing controversy about chemoprophylaxis in the Netherlands

The prescription of rifampicin to the household contacts of a primary patient with MD, as well as to the primary patient, is generally accepted practice in the United States and Great Britain.[38 39] The ultimate goal of this measure is to prevent secondary cases of MD. The beneficial effect of rifampicin on the prevention of secondary cases, however, has been demonstrated only in the case of meningitis caused by *Haemophilus influenzae*, but not for MD.[40]

It is very difficult, if not impossible, to give an exact definition of a secondary case of MD, because it is not possible to separate true secondary cases which were infected by the primary patient, from those which were infected by another source, e.g. another member of the family or even a source outside the household.[41] It is, therefore, better to speak of "associated cases" instead of "secondary cases".[42] The idea of preventing secondary or associated cases of MD by the use of chemoprophylaxis is based on the assumption of interrupting the transmission of the meningococci within the household by eliminating them from the nasopharynx of those household members who are carriers. In this respect, the prescription of chemoprophylaxis seems logical. As the primary source of infection of an associated case could also be a person outside the household, the strain might well be reintroduced into the household. Therefore, full protection by the use of chemoprophylaxis can never be guaranteed.

In the Netherlands it is left to the opinion of the attending physician whether or not to prescribe chemoprophylaxis.[43] During our nation-wide survey in the period 1989-1990, chemoprophylaxis was prescribed to 55% of the household members. In only 45% of these (i.e. in 25% of all household members), the prophylaxis was given according to an optimal regimen, whereas the primary patients were rarely included (Chapter 6). We conclude that the recommendations concerning the prescription of chemoprophylaxis in the Netherlands, compared with the standards in the United States and Great Britain, are inappropriate and should be more clearly formulated. Despite the fact that the beneficial effect of chemoprophylaxis on the prevention of secondary MD has never been proved, we do advocate its prescription. We have based our opinion on the results of a randomized trial of rifampicin for the prevention of secondary cases of meningitis due to *H. influenzae*, and on the circumstantial evidence of the preventive effect of chemoprophylaxis on the occurrence of secondary cases of MD.[40 44 45] If opponents of chemoprophylaxis are not convinced by these arguments, they should take the initiative for further research. A nation-wide randomized placebo-controlled clinical trial on the effect of rifampicin for the prevention of associated cases is to be preferred, but will encounter major organizational constraints. An alternative, and probably easier to perform design could be a field trial in two different areas of the country, in which rifampicin is prescribed to the household contacts of primary patients in one area. In both instances, the trial will need to run for a long time in order to allow for the occurrence of a sufficient number of associated cases to ensure enough discriminating power. It can be argued that in both study types the household itself should be taken as unit of measurement instead of individual household members, which necessitates an even longer time-span for the trial. Because meningococci rapidly develop resistance to rifampicin, it may be worthwhile to test the effect of other antimicrobial drugs in preventing secondary cases of MD (e.g. ceftriaxone or ciprofloxacin).[46-48]

Concluding remarks

Despite the fact that numerically speaking MD constitutes a relatively minor problem - in the Netherlands 300-500 cases per year - the high case-fatality and sequelae rates resulting from this disease continue to be a subject of considerable concern. Notwithstanding the scientific progress that has been made in the past era regarding our knowledge of *N. meningitidis*, it still remains obscure why the majority of individuals who acquire meningococci become carriers, and only a minority develop a life-threatening disease. The improvements in clinical care that have been made in the past four decades have not influenced the case-fatality rate of MD.[49] The only means to circumvent the problems caused by this serious disease is to prevent its occurrence by vaccination. All efforts should be directed towards the further development of a serogroup B meningococcal vaccine and the improvement of the existing serogroup A/C vaccine. Therefore, careful monitoring of the various epidemiological markers of meningococci must continue, with the aim of eventually achieving the development of a vaccine which will give optimal protection against meningococcal disease.

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SUMMARY

Summary

Meningococcal disease (meningitis and/or septicemia) is a serious illness. The case-fatality rate (CFR) is approximately 10%, and approximately 10% of the patients are left with serious sequelae. The causative organism, *Neisseria meningitidis* (or meningococcus), is capable of causing epidemics of meningococcal disease (MD). Since 1958, isolates of *N. meningitidis* recovered from patients with MD in the Netherlands have been forwarded to the Netherlands Reference Laboratory for Bacterial Meningitis (RLBM) of the University of Amsterdam and the National Institute for Public Health and Environmental Protection by almost all Dutch clinical microbiological laboratories. The isolates are stored at -70°C, and are constantly available for further research.

In the period between 1982 and 1986, a gradual increase in the incidence of MD was noted in the Netherlands, which seemed to stabilize in 1986. At the end of 1988, however, the number of cases increased further, and an epidemic of MD was feared.

Currently, it is not possible to prevent (an epidemic of) MD, because no vaccine is available against meningococci of serogroup B, which predominates in most industrialized countries. The development of such a vaccine is mainly based on the class 1 outer membrane protein (OMP) of the meningococcus. Epitopes of the class 1 OMP determine the subtype of the meningococcus.

In view of the continuing increase of the incidence of MD in the Netherlands, it was decided at the beginning of 1989 to start a nation-wide survey. The objectives of this study were to find possible explanations for the increase and to provide data which could assist the further development of a meningococcal serogroup B vaccine. Secondary goals were the assessment of the secondary attack rate of MD among the household contacts of primary patients, and the assessment of the prescription of chemoprophylaxis for the prevention of secondary cases. Data were collected from 1st April 1989 to 30th April 1990, co-ordinated by the Institute for Research in Extramural Medicine (EMGO Institute) of the Vrije Universiteit in Amsterdam. Data-collection was made possible thanks to the intense efforts of many Public Health Officers, the personnel of many clinical microbiological laboratories and attending physicians. For the further analysis of secular changes in the characteristics of *N. meningitidis* in the past 32 years, the extensive collection of meningococcal isolates of the RLBM was available.

This thesis describes the results of the epidemiological part of the survey. Serological research still continues, and will be presented later.

In Chapter 1 some general features of *N. meningitidis* and MD are summarized, and the objectives of the study are presented.

Chapter 2 presents the results of serotyping and subtyping of meningococcal isolates obtained during the period from 1980 to 1990 and of a sample of serogroup B isolates obtained between 1958 and 1975. From 1980 to 1990 the incidence of MD in the Netherlands gradually increased from 1.1 per 100.000 inhabitants in 1982 to 3.5 per 100.000 inhabitants in 1990. The increase could partially be explained by the

emergence of a meningococcal strain of a new phenotype: B:4:P1.4. This strain, which was not found in the Netherlands before 1980, accounted for 21% of all cases of MD in 1990. The increase is mainly due to meningococci of various phenotypes, which were already present in the Netherlands. This suggests the existence of additional explanations for the increase, which are not related to the meningococcus. Among serogroup B meningococci, the predominant serogroup in the Netherlands, many different subtypes were found, and between 1958 and 1990 gradual, but major changes in the distribution of subtypes occurred. From 1980 to 1990 a shift of MD to older patients was found, which could be partially explained by the emergence of new meningococcal phenotypes. Between 1980 and 1990 the proportion of meningococci isolated from blood alone increased, which might indicate an increase in the virulence of meningococci during this period. This increase, however, was not associated with specific phenotypes.

Chapter 3 describes the appearance of a sub-population of genetically closely related meningococci (clones) and the genotypic and phenotypic changes that occurred within this sub-population after its appearance. The genetic relationship of a number of meningococci, obtained by stratified sampling, was assessed by determining the electrophoretic type (ET) by means of multilocus enzyme electrophoresis. Around 1980 a new meningococcal clone (ET-24) was found in the Netherlands, which was homogenous with regard to the phenotype (B:4:P1.4). Until 1984 the clone remained stable, but later various new clones derived from the original clone. In addition, an increasing number of other serotypes and subtypes were found among the isolates of these clones, suggesting the exchange of genetic material between the various meningococci. The rapid changes in the serotype and subtype of genetically related meningococci necessitate a regular adaptation of a future vaccine which is based on the antigens which determine the serotype and subtype.

In addition to the serogroup, serotype and subtype, the lipooligosaccharide (LOS) immunotype is another major surface characteristic of the meningococcus. Chapter 4 describes the applicability of the whole-cell ELISA (WCE) with monoclonal antibodies (moabs) for the determination of the LOS immunotype. Despite the lack of specific moabs against the immunotypes L2 and L4, it was possible to develop an algorithm for the assignment of immunotypes on the basis of the reaction patterns of the isolates to a panel of 14 moabs. The immunotypes of 57 isolates, as determined by WCE, were in accordance with those obtained earlier by microprecipitation. In addition, the results from the WCE were reproducible. The distribution of immunotypes of meningococci isolated from patients with MD in the period 1989-1990 was presented. Meningococci of the serogroups B and C harboured a limited number of different immunotypes. Meningococcal immunotyping by WCE was found to be easily applicable, and provided the possibility of better definition of test strains for *in vitro* bactericidal assays and pathogenesis research.

In Chapter 5 the association between meningococcal surface characteristics and patient characteristics, on the one hand, and the occurrence of sequelae and fatalities on the other, was investigated. The meningococcal isolates, obtained in the period 1989-1990 from 562 patients with MD, were characterized with regard to their surface markers. Data on the patients were collected by means of a questionnaire. 8.5% of the patients who survived, recovered with sequelae. The occurrence of sequelae was only associated with the presence of septicemia. The CFR was 7.7%. In a multivariate logistic regression analysis age, gender and the clinical presentation were the most important determinants for a fatal outcome. Infants under the age of 6 months, patients from 10 to 19 years old, and patients over 50 years of age were found to be at greater risk for a fatal outcome compared to patients between the age of 6 months and 10 years (Odds Ratios respectively 5.3, 3.4 and 9.8). Comparing females with males, this Odds Ratio was 2.3. Patients with septicemia alone, or a combination of septicemia and meningitis, were at greater risk for a fatal outcome than patients suffering from meningitis alone (Odds Ratios respectively 2.3 and 3.1). In the bivariate analysis there were indications of an association between some meningococcal surface characteristics and the outcome of MD, but in the multivariate analysis these characteristics did not influence the outcome. This suggests that this bivariate relationship is confounded by patient characteristics.

In Chapter 6 the prescription of chemoprophylaxis to the household contacts of primary patients with MD is described with regard to the period 1989-1990. In the United States and Great Britain the prescription of chemoprophylaxis is accepted practice. In the Netherlands, however, the prescription of chemoprophylaxis is left to the opinion of the attending physician. During the study-period, 502 primary patients with MD were reported, and 5 secondary cases. The estimated secondary attack rate was 0.3%, which is similar to that in other countries. Chemoprophylaxis had been prescribed to 55% of the household contacts who completed the questionnaire. The prophylaxis was considered optimal (defined as the prescription of rifampicin or minocycline to all household contacts within 1 day after admission to hospital of the primary patient) in 46% of the contacts who had received prophylaxis, among which 1 secondary case. All meningococcal isolates obtained from a pair of consisting a primary and secondary case, were sensitive to rifampicin. It was concluded that the recommendations concerning chemoprophylaxis in the Netherlands, compared with standards elsewhere, are inappropriate and are in need of reappraisal. Based on results from this study and the literature, the prescription of chemoprophylaxis is recommended.

Due to the heterogeneity of meningococcal isolates and the gradual rate of increase in the incidence of MD from 1980 to 1990, the current situation in the Netherlands can best be described as hyperendemic, and there are no indications for the existence of an epidemic. The increase seems due to a combination of factors of the host and pathogen. The emergence of a new meningococcal sub-population

explains part of the rise. The serological section of the survey may give further clues about the influence of host factors on the increase, such as a decreased herd immunity or an increased migration of the Dutch population. The shift of MD to older patients, observed in the 1980s, could be explained by the appearance of new meningococcal strains. The extensive collection of meningococcal isolates of the RLBM is extremely suitable for further analyses of the relationship between changes in the age-distribution and the appearance of new meningococcal populations, for example by analysing the distribution of serotypes and subtypes in various birth-cohorts. From 1980 to 1990 an apparent increase in meningococcal virulence was noted, indicated by the increase in the proportion of strains obtained from blood alone, and the increased CFR compared to that of the period 1959-1981. This increased virulence could not be attributed to any specific meningococcal strain. A possible explanation might be the existence of a common, but not yet identified virulence factor, e.g. increased endotoxin release by the meningococcus.

In view of the occurrence of many different serotypes and subtypes among serogroup B meningococci, a multivalent serogroup B OMP vaccine will be needed in the Netherlands. This can also be concluded from the results of the trials with the first experimental OMP vaccines in other countries. The secular changes in the distribution of serotypes and subtypes necessitate regular adaptation of such a vaccine, and presumably vaccination of (parts of) the population with the adapted vaccine.

SAMENVATTING

Meningokokkenziekte (meningitis en/of sepsis) is een ernstige ziekte. Ongeveer 10% van de patiënten overlijdt en een zelfde percentage geneest met ernstige restverschijnselen. De verwekker, *Neisseria meningitidis*, of meningokok, is in staat epidemieën te veroorzaken. Vanaf 1958 worden door het merendeel van de Nederlandse klinisch microbiologische laboratoria stammen van *N. meningitidis* die geïsoleerd zijn bij patiënten met meningokokkenziekte, ingezonden naar het Nederlands Referentie Laboratorium voor Bacteriële Meningitis (RLBM) van de Universiteit van Amsterdam en het Rijksinstituut voor Volksgezondheid en Milieuhygiëne. Deze meningokokken worden ingevroren en zijn steeds beschikbaar voor verder onderzoek.

Tussen 1982 en 1986 werd in Nederland een geleidelijke stijging van de incidentie van meningokokkenziekte geconstateerd, die in 1986 tot staan leek te zijn gekomen. Eind 1988, echter, vond een verdere stijging plaats en men vroeg zich af, of er mogelijk sprake was van een (beginnende) epidemie.

Op dit moment is het nog niet mogelijk (een epidemie van) meningokokkenziekte te voorkómen, aangezien geen vaccin beschikbaar is tegen meningokokken van serogroep B, de meest voorkomende serogroep in geïndustrialiseerde landen. De ontwikkeling van een dergelijk vaccin is gebaseerd op de subtype bepalende epitopen van het klasse 1 buitenmembraaneiwit van de meningokok.

Vanwege de verder stijgende incidentie van meningokokkenziekte in Nederland werd begin 1989 besloten tot het uitvoeren van een landelijk onderzoek. Doel van dit onderzoek was het geven van een mogelijke verklaring voor de stijging en het verzamelen van gegevens die een bijdrage konden leveren aan de verdere vaccinontwikkeling. Een nevendoel was het bepalen van de "secondary attack rate" van meningokokkenziekte onder huisgenoten van primaire patiënten en het inventariseren van het gebruik van chemoprofylaxe ter voorkoming van secundaire gevallen. De gegevensverzameling vond plaats in de periode van 1 april 1989 tot en met 30 april 1990 en werd gecoördineerd door het Instituut voor Extramuraal Geneeskundig Onderzoek (EMGO-Instituut) van de Vrije Universiteit te Amsterdam. De gegevensverzameling werd mogelijk gemaakt door de meer dan voortreffelijke medewerking van vele artsen en sociaal verpleegkundigen van de verschillende Nederlandse gezondheidsdiensten, medewerkers van de microbiologische laboratoria en behandelend specialisten. Bovendien kon gebruik gemaakt worden van de collectie meningokokken van het RLBM.

Dit proefschrift beschrijft de resultaten van het epidemiologische deel van het onderzoek. Het serologisch onderzoek is nog in volle gang en zal later gepubliceerd worden.

In Hoofdstuk 1 worden enige algemene aspecten van *N. meningitidis* en meningokokkenziekte besproken en worden de doelstellingen van het onderhavige onderzoek geformuleerd.

In Hoofdstuk 2 worden de resultaten gepresenteerd van de sero- en subtypering van meningokokken uit de periode 1980-1990 en van een steekproef van meningo-

kokken van serogroep B uit de periode 1958-1975. In de periode 1980-1990 steeg de incidentie van meningokokkenziekte geleidelijk van 1,1 per 100.000 inwoners in 1982 tot 3,5 per 100.000 inwoners in 1990. Deze stijging kon ten dele verklaard worden door de opkomst van een meningokokkenstam met een nieuwe serotype-subtype combinatie (fenotype): B:4:P1.4. Deze stam, die vóór 1980 nog niet in Nederland was gesignaleerd, was verantwoordelijk voor 21% van alle gevallen van meningokokkenziekte in 1990. Het merendeel van de stijging wordt veroorzaakt door meningokokken van verschillende fenotypen, die al langer in Nederland aanwezig waren. Dit wijst op het bestaan van bijkomende, buiten de meningokok gelegen oorzaken voor de stijging. Binnen serogroep B, de meest voorkomende serogroep in Nederland, kwamen vele verschillende subtypen voor en tussen 1958 en 1990 vonden geleidelijke, maar belangrijke veranderingen plaats in de subtypeverdeling van meningokokken van deze serogroep. Van 1980 tot 1990 was er een opvallende verschuiving in de leeftijdsverdeling van de patiënten met meningokokkenziekte van jongere naar oudere leeftijdscategorieën, die deels verklaard kon worden door de opkomst van nieuwe meningokokkenstammen. Tussen 1980 en 1990 steeg het percentage meningokokken die alleen uit het bloed van patiënten geïsoleerd waren, hetgeen zou kunnen wijzen op een toename van de virulentie van de verschillende meningokokken in de beschreven periode. Deze stijging was niet geassocieerd met bepaalde fenotypen.

Hoofdstuk 3 bevat een beschrijving van de opkomst van een subpopulatie van meningokokken met dezelfde genetische achtergrond (klonen) en de veranderingen in het genotype en fenotype die zich in de loop der tijd hebben voorgedaan binnen deze subpopulatie. Van een aantal door een gestratificeerde steekproef verkregen meningokokken werd het "electrophoretic type" (ET) bepaald door middel van "multilocus enzyme electrophoresis" als kenmerk voor de genetische achtergrond. Rond 1980 werd in Nederland een nieuwe kloon (ET-24) aangetroffen die homogeen was wat betreft het fenotype (B:4:P1.4). Tot 1984 bleef deze kloon stabiel, maar daarna ontstonden hieruit verschillende andere klonen. Bovendien werden binnen deze nieuwe klonen in toenemende mate andere serotypen en subtypen gevonden, hetgeen wijst op de uitwisseling van genetisch materiaal tussen de verschillende meningokokken. De snelle veranderingen in het serotype en subtype van meningokokken met dezelfde genetische achtergrond maken een regelmatige aanpassing noodzakelijk van een toekomstig vaccin dat gebaseerd is op antigenen die het serotype en subtype bepalen.

Naast de serogroep, het serotype en subtype is het lipo-oligosaccharide (LOS) immunotype een belangrijk oppervlaktekenmerk van de meningokok. Hoofdstuk 4 bevat een beschrijving van de toepasbaarheid van de "whole-cell ELISA" (WCE) voor het bepalen van het LOS immunotype van de meningokok met behulp van monoklonale antilichamen. Ondanks het ontbreken van specifieke monoklonale antilichamen tegen de immunotypen L2 en L4 bleek het mogelijk een algoritme te ontwikkelen waarmee de meeste immunotypen bepaald konden worden. Dit gebeurde aan de hand van het reactiepatroon van de isolaten met de verschillende monoklonalen. Van

57 isolaten was het immunotype al eens eerder bepaald met behulp van een andere, meer bewerkelijke methode. De in de WCE bepaalde immunotypen van deze 57 isolaten kwamen goed overeen met de eerder bepaalde immunotypen. De resultaten verkregen in de WCE bleken bovendien goed reproduceerbaar. De verdeling van immunotypen van meningokokken geïsoleerd bij patiënten met gegeneraliseerde meningokokkenziekte in de periode 1989-1990 werd gepresenteerd. Binnen de serogroepen B en C komt een beperkt aantal verschillende immunotypen voor. Immunotypering van meningokokken is eenvoudig uitvoerbaar in de WCE. Met behulp van immunotypering wordt een betere definiëring van meningokokken mogelijk gemaakt ten behoeve van verder pathogenetisch en immunologisch onderzoek.

In Hoofdstuk 5 werd nagegaan, in hoeverre oppervlaktekenmerken van de meningokok en patiëntkenmerken geassocieerd zijn met het optreden van restverschijnselen of een dodelijk ziektebeloop. Hiertoe werden de meningokokken, die geïsoleerd werden bij 562 patiënten met meningokokkenziekte in de periode 1989-1990, gekarakteriseerd. Patiëntgegevens werden verkregen door middel van een vragenlijst. 8,5% van de patiënten herstelde met restverschijnselen. Het optreden van restverschijnselen was alleen geassocieerd met het voorkomen van sepsis. De letaliteit was 7,7%. In een multivariate logistische regressie-analyse bleken leeftijd, geslacht en klinische presentatie de belangrijkste determinanten voor een fataal ziektebeloop. Zuigelingen jonger dan 6 maanden, patiënten van 10-19 jaar en patiënten ouder dan 50 jaar bleken een verhoogd risico te hebben op overlijden vergeleken met patiënten in de leeftijd tussen 6 maanden en 10 jaar (Odds Ratio's respectievelijk 5,3, 3,4 en 9,8). Voor vrouwen vergeleken met mannen bedroeg deze Odds Ratio 2,3. Patiënten met alleen sepsis of een combinatie van sepsis en meningitis hadden een verhoogd risico op overlijden vergeleken met patiënten met alleen meningitis (Odds Ratio's respectievelijk 2,3 en 3,1). Hoewel er in de bivariate analyse aanwijzingen gevonden werden voor een associatie tussen enige stamkenmerken van de meningokok en het ziektebeloop, bleken deze in de multivariate analyse het beloop niet te beïnvloeden. Dit wijst op vertekening van de bivariate relatie door patiëntkenmerken.

In Hoofdstuk 6 wordt een inventarisatie gegeven van het voorschrijven van chemoprofylaxe aan de huisgenoten van primaire patiënten met meningokokkenziekte in de periode 1989-1990. In de Verenigde Staten en Groot Brittannië is het voorschrijven van chemoprofylaxe algemeen geaccepteerd. In Nederland wordt het al dan niet voorschrijven van chemoprofylaxe aan de behandelend specialist overgelaten. Gedurende de onderzoeksperiode werden 502 primaire patiënten met meningokokkenziekte gemeld en 5 secundaire gevallen. De geschatte "secondary attack rate" bedroeg 0,3%, hetgeen overeenkomt met cijfers uit andere landen. Aan iets meer dan de helft van de ondervraagde gezinscontacten werd enige vorm van chemoprofylaxe voorgeschreven. Van de huisgenoten die profylaxe kregen voorgeschreven, kreeg iets minder dan de helft optimale profylaxe (gedefinieerd als het gebruik van rifampicine of minocycline door alle huisgenoten, gestart ten hoogste 1 dag na opname van de

primaire patiënt), waaronder 1 secundair geval. Alle bij de secundaire en bijbehorende primaire patiënten geïsoleerde meningokokken waren gevoelig voor rifampicine. Geconcludeerd werd, dat de richtlijnen ten aanzien van chemoprofylaxe in Nederland, gerekend naar maatstaven elders, onvolledig zijn en dienen te worden bijgesteld. Mede op grond van literatuurgegevens wordt het voorschrijven van chemoprofylaxe bepleit.

Gezien de heterogeniteit van de meningokokken en het geleidelijke beloop van de incidentiestijging van meningokokkenziekte van 1980 tot 1990, kan de huidige situatie in Nederland het beste als hyperendemisch beschreven worden, en is er geen sprake van een epidemie. De oorzaak van de stijging lijkt gelegen in een combinatie van gast- en gastheerfactoren. De opkomst van een nieuwe meningokokkenpopulatie verklaart slechts een deel van de stijging. Over het aandeel van gastheerfactoren, zoals een verlaagde immuniteit in de algemene bevolking en een toegenomen migratie van de Nederlandse bevolking, kan het serologische deel van het onderzoek mogelijk uitsluitsel geven. De verschuiving in de leeftijdsverdeling die plaats vond in de jaren tachtig, kon verklaard worden door de opkomst van nieuwe meningokokken stammen. De uitgebreide meningokokkencollectie van het RLBM leent zich uitstekend voor het doen van een uitgebreidere analyse van het verband tussen de veranderende leeftijdsverdeling en de opkomst van nieuwe meningokokkenpopulaties, bijvoorbeeld door de analyse van de leeftijdsspecifieke verdeling van sero- en subtypen in verschillende geboortecohorten. Van 1980 tot 1990 lijkt er sprake te zijn van een toegenomen virulentie van de diverse meningokokken, hetgeen gesuggereerd wordt door een toename van het percentage meningokokken die geïsoleerd zijn uit alleen het bloed, en een verhoogde letaliteit ten opzichte van de periode 1959-1981. Deze verhoogde virulentie kon niet toegeschreven worden aan de opkomst van een bepaalde stam. Mogelijk is er sprake van een gemeenschappelijke, maar nog niet gedetecteerde virulentiefactor, zoals bijvoorbeeld een verhoogde endotoxine-afgifte door de meningokok.

Gezien het voorkomen van vele verschillende subtypen binnen serogroep B meningokokken zal een toekomstig vaccin voor Nederland multivalent moeten zijn. Dit kan ook geconcludeerd worden uit de resultaten van de experimenten met de eerste vaccins in andere landen. Gezien de periodieke veranderingen die optreden in de verdeling van subtypen zal een dergelijk vaccin van tijd tot tijd aangepast moeten worden en zal er mogelijk opnieuw gevaccineerd moeten worden met het aangepaste vaccin.

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